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# Vascular KATP Channel Modulation by S-Glutathionylation: A Novel Mechanism for Cellular Response to Oxidative Stress

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**VASCULAR K<sub>ATP</sub> CHANNEL MODULATION BY S-GLUTATHIONYLATION: A  
NOVEL MECHANISM FOR CELLULAR RESPONSE TO OXIDATIVE STRESS**

by

YANG YANG

Under the Direction of Dr. Chun Jiang

**ABSTRACT**

The K<sub>ATP</sub> channels play an important role in the membrane excitability and vascular tone regulation. Previous studies indicate that the function of K<sub>ATP</sub> channels is disrupted in oxidative stress seen in a variety of cardiovascular diseases, while the underlying mechanism remains unclear. Here, we demonstrate S-glutathionylation to be a modulation mechanism underlying the oxidant-mediated vascular K<sub>ATP</sub> channel inhibition, the molecular basis for the channel inhibition and the alleviation of the channel inhibition by vasoactive intestinal peptide (VIP). We found that an exposure of isolated mesenteric rings to H<sub>2</sub>O<sub>2</sub> impaired the K<sub>ATP</sub> channel-mediated vascular dilation. In whole-cell recordings and inside-out patches, micromolar H<sub>2</sub>O<sub>2</sub> or diamide caused a strong inhibition of the vascular K<sub>ATP</sub> channel (Kir6.1/SUR2B) in the presence, but not in the absence, of glutathione (GSH), indicating S-glutathionylation. By co-expressions of Kir6.1 or Kir6.2 with SUR2B subunits, we found that the oxidant sensitivity of the K<sub>ATP</sub> channel relied on the Kir6.1 subunit. Systematic mutational analysis revealed three cysteine residues (Cys43, Cys120 and Cys176) to be important. Among them, Cys176 was prominent, contributing to >80% oxidant sensitivity. Biochemical pull-down assay with biotinylated glutathione ethyl ester (BioGEE) showed that mutations of Cys176 impaired the oxidant-induced incorporation of GSH

to the Kir6.1 subunit. Simulation modeling of Kir6.1 S-glutathionylation revealed that after incorporation to residue 176, the GSH moiety occupied a space between slide helix and two transmembrane helices. This prevented the necessary conformational change of the inner helix for channel gating, and retained the channel in its closed state. VIP is a potent vasodilator, and is shown to have protective role against oxidative stress. We found that the channel was strongly augmented by VIP and the channel activation relied on PKA phosphorylation. These results therefore indicate that 1) the vascular  $K_{ATP}$  channel is strongly inhibited in oxidative stress, 2) S-glutathionylation underlies the oxidant-mediated  $K_{ATP}$  channel inhibition, 3) Cys176 in the Kir6.1 subunit is the major site for S-glutathionylation, and 4) the Kir6.1/SUR2B channel is activated in a PKA-dependent manner by VIP that has been previously shown to alleviate oxidative stress.

INDEX WORDS: Kir6.1, SUR2B, ATP-sensitive  $K^+$  channels,  $K_{ATP}$  channel, Vascular tone, Oxidative stress, S-glutathionylation, Structural modeling, Protein kinase A, Vasodilators, Vasoconstrictors, Vasoactive intestinal peptide, Reactive oxygen species.

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MECHANISM FOR CELLULAR RESPONSE TO OXIDATIVE STRESS

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YANG YANG

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Georgia State University

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## ABBREVIATIONS

2-PDS	2-pyridinedisulfides
2-DTP	2, 2'-dithiodipyridine
ABC	ATP binding cassette
AC	adenylyl cyclase
AMP	adenosine monophosphate
ADP	adenosine diphosphate
ATP	adenosine triphosphate
ANOVA	analysis of variance
BioGEE	biotinylated glutathione ethyl ester
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
cPKA	catalytic subunit of PKA
DIA	diamide
DAG	diacylglycerol
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's Modified Eagle Medium
DTNP	2, 2'-dithiobis-5-nitropyridine
EGTA	ethylene glycol-bis- $\beta$ -aminoethyl ether-N,N,N',N'-tetraacetic acid
FSK	forskolin
GFP	green fluorescent protein
Glib	glibenclamide
GPCR	G protein coupled receptors



Grx	glutaredoxin
GSH	reduced glutathione
GSSG	oxidized glutathione
H	hill coefficient
HEK	human embryonic kidney cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
EC <sub>50</sub>	concentration for 50% inhibition
IP <sub>3</sub>	inositol-1, 4, 5-triphosphate
Isop	isoproterenol
K <sub>ATP</sub>	ATP-sensitive K <sup>+</sup>
KCO	K <sup>+</sup> channel opener
Kir	inward rectifier K <sup>+</sup> channel
Kir6.x	members in the sixth subfamily of inward rectifier K <sup>+</sup> channels
Kir6.2ΔC36	inward rectifier K <sup>+</sup> channel 6.2 with 36 amino acids truncated
K <sub>NDP</sub>	Nucleotide diphosphate activated K <sup>+</sup> channel
KO	gene knockout
K <sub>v</sub>	voltage-gated K <sup>+</sup> channel
LC-CoA	long chain acyl CoA
MLCK	myosin light chain kinase
NBD	nucleotide bonding domain
NDP	nucleotide diphosphate
NO	nitric oxide
O	open state

PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDB	protein database
PE	phenylephrine
Pin	pinacidil
PIP <sub>2</sub>	phosphatidylinositol biphosphate
PIPES	piperazine-N,N'-bis-2-2-ethanesulfonic acid
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PSS	physiological saline solution
P <sub>O</sub>	open-state probability
RT-PCR	reverse transcription polymerase chain reaction
RIPA buffer	Radio-Immunoprecipitation Assay buffer
SDS	sodium dodecyl sulfate
S.E.	standard error
SR	sarcoplasmic reticulum
SUR	sulphonylurea receptor
TM1	the first transmembrane segment in Kir
TM2	the second transmembrane segment in Kir
TMD	transmembrane domain
TMH	transmembrane helix
TRP	transient potential channels

VDCC	voltage-dependent $\text{Ca}^{2+}$ channel
VIP	vasoactive intestinal polypeptide
VSM	vascular smooth muscle
WT	wild type

## 1. SPECIFIC AIMS AND HYPOTHESES

Several reactive species are produced under pathological and pathophysiological conditions, such as reactive oxygen species (ROS) and reactive carbonyl species (RCS). Oxidative stress takes place when the production of these reactive species becomes long-lasting and exceeds the cellular anti-oxidant defense systems, which is seen in inflammation, diabetes, cardiovascular disease, neurodegenerative disorders, etc. The reactive species can modify proteins, lipids and DNAs, causing cellular dysfunction and structural damages. In vasculatures, the reactive species, especially ROS, can be released from damaged tissues, neutrophils, macrophages, and vascular endothelium. Plasma membranes and membrane proteins of the endothelial cells and smooth muscle cells (SMCs) are the primary targets of ROS. Some of these membrane proteins are critical for membrane potential regulation. Dysfunctions of these proteins can result in depolarization (Liu and Gutterman, 2002a; Oelze et al., 2006). Prolonged depolarization triggers  $\text{Ca}^{++}$  influx through the voltage-gated  $\text{Ca}^{++}$  channels, and facilitates the  $\text{Ca}^{++}$  release from intracellular  $\text{Ca}^{++}$  store in SMCs (Anzai et al., 2000; Wray et al., 2005). The elevated cytosolic  $\text{Ca}^{++}$  further causes blood vessel contraction resulting in vascular dysfunction and activation of other downstream effectors, including NADPH oxidase (NOX), some of which are known to be sensitive to  $\text{Ca}^{++}$  and PKC (Brandes et al., 2010; Guzik et al., 2008; Hink et al., 2001; Wendt et al., 2005; Zhang et al., 2008). Consequently, a cascade of events is initiated leading to further depolarization and more ROS production (al-Mehdi et al., 1997; Lyle and Griendling, 2006).

In SMCs, the membrane potential is primarily regulated by the ATP-sensitive  $\text{K}^{+}$  ( $\text{K}_{\text{ATP}}$ ) channel (Quayle et al., 1997; Teramoto, 2006), while the  $\text{K}_{\text{ATP}}$  channel is regulated by several metabolites, vasodilators and vasoconstrictors. The activation of this channel lowers the

membrane excitability, reduces the  $\text{Ca}^{++}$  influx, and may protect the cells from oxidative stress (Quayle et al., 1997). Therefore, information about the  $\text{K}_{\text{ATP}}$  channel activity during oxidative stress is critically important in the understanding of vascular responses to oxidative stress, which however remains unknown. A number of **questions remain open**: For example, is the vascular  $\text{K}_{\text{ATP}}$  channel a target of oxidative stress? How does the reactive species modulate  $\text{K}_{\text{ATP}}$  channel activity? What is the molecular mechanism underlying the modification? Does this modification lead to vascular dysfunction? What protein domains and specific amino acid residues of  $\text{K}_{\text{ATP}}$  are responsible for this modification? How does the activation of the  $\text{K}_{\text{ATP}}$  channel reduce the membrane excitability and protect the cells against oxidative stress? Can the channel be activated by endogenous vasodilators that play a protective role in oxidative stress? To address some of these questions, a series of experiments is proposed with the objective to understand the molecular and cellular basis for the modulation of the vascular  $\text{K}_{\text{ATP}}$  channel in oxidative stress. The **specific aim** of this dissertation thus was to demonstrate whether and how the  $\text{K}_{\text{ATP}}$  channels are modulated in oxidative stress. Three **specific hypotheses** were tested:

1. **The vascular  $\text{K}_{\text{ATP}}$  channel is a target of ROS and other oxidants via the protein post-translational modification mechanism.**
2. **This post-translational modification mechanism occurs on the cysteine residues located in the critical domains of the vascular  $\text{K}_{\text{ATP}}$  channel and structurally affects the channel gating.**
3. **VIP, a peptide hormone that has protective role against oxidative stress, affects the activity of vascular  $\text{K}_{\text{ATP}}$  channels via a specific intracellular signaling pathway.**

## **2 GENERAL INTRODUCTION**

### **2.1 Vascular tone regulation**

#### **2.1.1 Overview**

Vascular tone in the mammalian system is dynamically regulated by neuronal, hormonal and local metabolic mechanisms that are targeted at the heart and vasculatures. Disruption of this regulation leads to cellular dysfunction and even severe diseases including ischemia, hyperemia, shock and hypertension (Gutterman et al., 2005; Webb, 2003). Blood vessels are made of endothelial cells and smooth muscle cells (SMCs). The endothelial cells form the blood vessel lumen, while the SMCs wrap the endothelial cells and generate force to constrict or dilate the blood vessels. Acting on either the vascular endothelium, smooth muscle or both, the regulation of vascular tone is achieved by a complicated signaling network involving circulating hormones, neurotransmitters and metabolites (Liu and Gutterman, 2009). The regulation of vascular tone is often altered under pathological conditions. For example, hypertension may occur when the SMCs cannot relax in response to vasodilators. In sepsis, the resistant arteries are dilated so vastly that the blood pressure cannot be maintained by common vasoconstrictors. In addition, the dysfunction of blood vessels can also be seen in diabetes, known to be the major cause of death as a result of the development of diabetic vascular complications. The understanding of vascular tone regulation therefore has a major impact on the treatment for these diseases.

#### **2.1.2 Control of the vascular tone by membrane excitability**

Vascular SMCs are excitable cells. Their membrane potentials are critical in determining the smooth muscle contractility (Wray et al., 2005). Membrane depolarization mediated by the closure of  $K^+$  channels leads to the activation of voltage-dependent  $Ca^{++}$  channels (VDCC). The

$\text{Ca}^{++}$  influx through these VDCC raises the intracellular  $\text{Ca}^{++}$  concentration. The  $\text{Ca}^{++}$ -induced  $\text{Ca}^{++}$  release is then activated. A rise in intracellular  $\text{Ca}^{++}$  thus allows them to bind to calmodulin, forming a  $\text{Ca}^{++}$ -calmodulin complex. This complex further binds to myosin light-chain kinase (MLCK) and activates it. Activated MLCK thus phosphorylates the myosin light-chain (Herring et al., 2006), leading to the enhanced interaction of myosin with actin filaments, and smooth muscle contraction. (Bolton, 2005; Webb, 2003; Wray et al., 2005).

On the other hand, hyperpolarization reduces the activity of the VDCC, lowers the intracellular  $\text{Ca}^{++}$  levels, and eventually results in the relaxation of vascular SMCs. Since the myosin phosphorylation is a critical step mediating the vasoconstriction, an increase in the myosin light-chain phosphatase activity, that dephosphorylates the myosin light-chain is also an important mechanism to cause the relaxation of vascular SMCs cells (Guibert et al., 2008; Wray et al., 2005).

## **2.2 Kir channel superfamily**

### **2.2.1 Kir channel overview**

$\text{K}^{+}$  channels play an important role in membrane potential regulations in virtually all cells. In excitable cells, the membrane excitability is regulated specifically by  $\text{K}^{+}$  channels.  $\text{K}^{+}$  channels are grouped into 4 super-families: voltage-gated ( $\text{K}_V$ ) channels,  $\text{Ca}^{++}$ -activated  $\text{K}^{+}$  ( $\text{K}_{Ca}$ ) channels, two-pore domain  $\text{K}^{+}$  (TASK) channels and inward rectifier  $\text{K}^{+}$  (Kir) channels.

$\text{K}_V$  channels are mainly expressed in excitable cells, contributing to the action potential repolarization and shaping the frequency of action potentials.  $\text{K}_{Ca}$  channels consist of large-conductance (BK) channels and small-conductance (SK) channels, both of which are regulated by the intracellular  $\text{Ca}^{++}$ . TASK  $\text{K}^{+}$  channels are also known as “leak  $\text{K}^{+}$  channels”, mainly contribute to the maintaining the resting membrane potential of the cells. Kir channels were

named by the special feature of this group of channels:  $K^+$  move through these channels into the cells more easily than going out of the cells. They are divided into 7 subfamilies (Kir1.1 to Kir7.1) (Hibino et al., 2010). Some suggest that they belong to 4 distinct groups (Hibino et al., 2010). The first group is the classical Kir channels (Kir2.x), which is considered as constantly activated Kir channels. The second group is the G protein-coupled Kir (GIRK) channels, also known as Kir3.x channels. This group of Kir channels can be directly regulated by  $G\beta\gamma$  proteins. The third group is called the ATP-sensitive  $K^+$  channel (Kir6.x), activity of which, in general, is associated with the cellular metabolic status. The Kir 6.x subunits do not form functional channels by themselves. A special regulatory subunit i.e., the sulfonylurea receptor (SUR), is required for the surface expression of the channels and their functional activity. Because of this feature, these channels are often listed as a distinct channel family rather than a group of Kir channels. The last group of Kir channels is referred to as  $K^+$  transport channels. This is the biggest group in the Kir family, which includes Kir1x, Kir4x, Kir5.1 and Kir7.1 (Bichet et al., 2003; Hibino et al., 2010).

### **2.2.2 Kir channel structure**

Several crystal structures of Kir channels have been resolved, including bacterial KirBac1.1 and the recent chicken Kir2.2 (Kuo et al., 2005; Kuo et al., 2003; Tao et al., 2009). It is clear that the core structure of Kir channel is conserved, sharing a high similarity with other  $K^+$  channels including KcsA, MthK and  $K_v$  channels. The core domain of Kir channels comprises of 1) two transmembrane helices named outer helix (TM1) and inner helix (TM2), 2) the pore loop or selectivity filters, and 3) the slide helix. The slide helix is distinct in the Kir channels, and does not exist in other  $K^+$  channels. The selectivity filter is highly conserved among  $K^+$  channels, with a signature sequence of T-XG-Y(F)-G (T, threonine; X, any amino acid, G, glycine; Y,



tyrosine; F, phenylalanine). Because the Kir channels are not voltage sensitive, they do not have other transmembrane domains e.g., the S1-S4 voltage paddle. The C- and N-termini form intracellular domains which vary among different Kir channels.

Several residues of the transmembrane helices have been pointed out in the structure of Kir channel to play central roles in the channel gating (Kuo et al., 2005; Kuo et al., 2003; Tao et al., 2009). One key residue in the TM2 is phenylalanine (Phe146 in KirBac1.1), which is located in the narrowest neck of the ion conductance pathway. When the channel enters the closed state, the bulk residue blocks the ion conductance pathway and prevents the ions from passing through. A glycine residue (Gly83 in KirBac1.1) is critical, that is known to form a flexible point in  $\alpha$ -helix. When the channel enters its open state, the TM2 helix bends at the glycine residue, moving the side-chain of the blocking Phe146 away from the center. This movement allows the ions to pass through the channel. A cysteine residue (Cys166 in Kir6.2) is known to play an important role in Kir6.2 channel gating. The concentration of the residues critical for Kir channel gating at a narrow region of TM2 suggests that other channel regulation mechanisms may be also located in the inner mouth of the ion-conductive pathway. Taking the advantage of the bacterial and chicken Kir channel crystal structures, we modeled our Kir6.1 subunit of the  $K_{ATP}$  channel to study the effect of S-glutathionylation on the gating of the  $K_{ATP}$  channels.

### **2.2.3 Inwardly rectifying feature**

The Kir channels feature the ability to let more  $K^+$  ions enter the cell than leaving from the cell. Two intracellular substances ( $Mg^{++}$ , and polyamines) determine the inward rectification, which interact with the lining of the channel pore, physically block the channel pore intracellularly. Such a blockage relies specifically on a negatively charged residue (Asp172 of

the strong rectifier Kir2.1 channel) in the TM2 helix (Ficker et al., 1994; Wible et al., 1994), which is also critical for pH sensitivity and the unitary conductance in Kir4.1 (Xu et al., 2000) . Among the Kir channel superfamily, different Kir channels show varying levels of inward rectification. Kir2.x and Kir3.x are considered as strong rectifiers, Kir4.x as intermediate, while Kir1.x and Kir6.x as weak rectifiers. Consistently, Asp at TM2 helix is found in the strong rectifiers and this residue is replaced by an uncharged Asparagine (Asn) in weak rectifiers. Site-directed mutagenesis has shown that if the Asp in Kir1.1 is changed to Asn experimentally, the weak rectifier Kir1.1 becomes a strong rectifier, showing an increase in binding affinity for  $Mg^{++}$  (Ficker et al., 1994; Wible et al., 1994). The channels studied in my dissertation were Kir6.x (Kir6.1 and Kir6.2) channels, which have weak inward rectification.

#### **2.2.4 Kir channel blockers.**

Specific Kir channel blockers are not available. However,  $Ba^{++}$  is often used in research as a relatively specific blocker for Kir channels. The specificity is dependent on the concentration of these ions. Micromolar concentration of  $Ba^{++}$  is able to block Kir channel but not other channels, whereas higher concentration can also affect  $K_V$  channels. In contrast, the  $K_V$  channel inhibitor tetraethylammonium (TEA) and 4-aminopyridine (4-AP) have very little effects on Kir channels (Hibino et al., 2010; Oliver et al., 1998).

### **2.3 ATP-sensitive $K^+$ or $K_{ATP}$ channels**

#### **2.3.1 Discovery of $K_{ATP}$ channel**

The  $K_{ATP}$  channel activity was first recorded in cardiac muscles (Noma, 1983). Later, the  $K_{ATP}$  currents were also identified in other tissues including: pancreatic  $\beta$ -cells (Ashcroft et al., 1984; Cook and Hales, 1984), skeletal muscle (Spruce et al., 1987), vascular SMCs (Standen et

al., 1989), and neurons (Ashford et al., 1988). Most  $K_{ATP}$  channels are sensitive to the intracellular ATP/ADP ratio. They are inhibited by ATP and activated by NDP e.g., MgADP. The vascular isoform of  $K_{ATP}$  channel is activated by intracellular NDP but has very little sensitivity to ATP. By sensing the ATP and NDP levels, these  $K_{ATP}$  channels are able to couple the cellular metabolism to membrane excitability.

The successful cloning of  $K_{ATP}$  channel was not achieved until 1995 (Inagaki et al., 1995). Initially, the Kir subunit was cloned from rat pancreatic islets but this clone did not show any functional current. Only after the cloning of the SUR subunit (Aguilar-Bryan et al., 1995), the functional  $K_{ATP}$  currents were recorded in the expression system.

### **2.3.2 SUR regulatory subunit**

The SUR belongs to the “C” branch of the ATP binding cassette (ABC) protein superfamily, which also includes multidrug resistance proteins (MRPs) and cystic fibrosis conductance regulator (CFTR/ABCC7). Typical ABC transporters have two clusters of membrane spanning domains (MSDs), each containing 6 transmembrane helices and 2 nucleotide binding (NBD) domains, with high-affinity ATP binding motifs. The NBDs are located intracellularly and link adjacent MSDs (Matsuo et al., 2000).

The SURs are considered as “long” ABCC proteins, with three MSDs and two NBDs. Each MSD1 and MSD2 is made of 6 transmembrane helices while MSD0 has five transmembrane helices. Therefore, SUR is a big transmembrane protein with 17 membrane spanning domains and an N-terminus located on the extracellular side.

The two NBDs in the SUR subunit can bind and hydrolyze ATP. The hydrolysis of ATP by NDB generates energy towards the conformational change of the MSD domains, which may further produce a force for the gating the pore forming Kir subunit. The catalytic sites of NBDs

are made up of Walker-A-motif of one NBD domain and the ABC signature sequence motif (C-motif) of the other NBD. Besides, several other conservative motifs exists in the NBD1 and NBD2, including Q-Loop, D-loop and H-loop (Deeley et al., 2006).

Without the major domains, the TMD0 domain of the SUR subunit itself could greatly enhance the C-terminus truncated form of Kir6.2 channels activity. The combination of TMD0 and Kir6.2 thus is known as “mini-K<sub>ATP</sub> channel” (Babenko and Bryan, 2002, 2003; Chan et al., 2003). The mini-K<sub>ATP</sub> channel cannot be regulated by sulfonylureas, Mg<sup>++</sup>-NDP or channel openers but it retains the ATP sensitivity. These data suggest that the ATP binding site is probably located in the domains of mini-K<sub>ATP</sub> channel, and a complete SUR subunit is necessary for sulfonylurea, K<sup>+</sup> channel opener (KCO) and NDP to have effect on the channel activity (Babenko and Bryan, 2002, 2003).

The SUR has two isoforms (*abcc8*/SUR1 and *abcc9*/SUR2). The SUR2 mRNA can undergo alternative splicing and generates SUR2A and SUR2B, which differ only at the last 42 amino acids in the C-terminus (C42). Interestingly, the C42 of SUR2B shares a pretty high similarity with that of SUR1 (Isomoto et al., 1996).

The interactions of Kir and SUR subunits, which lead to K<sub>ATP</sub> channel gating, are still elusive. It is suggested that the TMD0 domain of the SUR subunit interacts with the sliding helix, outer helix and N-terminus of Kir subunit (Babenko and Bryan, 2002, 2003). The adjacent L0 linker may bridge the Kir6.2/MSD0 with the core SUR (Fang et al., 2006). Lately, an ED domain of SUR2A, containing 15 negatively charged amino acid residues, has been identified that acts as a transducer that links the conformational change of sulfonylurea receptor to gating of Kir6.2 (Karger et al., 2008). When the SUR undergoes conformational change upon the stimulation of channel opener, it generates a lateral tension force that pulls the slide helix. The

lateral movement of the slide helix further causes the conformational change of the inner pore-forming helix and moves the gate away to open the channel.

### **2.3.3 Tissue distribution and molecular identity**

$K_{ATP}$  channels have been identified in a number of tissues including pancreatic  $\beta$  cells, SMCs, heart muscle cells, and neurons: Kir6.2/SUR1 isoform is expressed in the  $\beta$  cells, where the closure of Kir6.2/SUR1 by an increase ATP/ADP ratio leads to the membrane depolarization of  $\beta$ -cells and insulin secretion. Kir6.1/SUR2B isoform is mainly expressed in the SMCs, contributing to the vascular tone regulation. The opening of Kir6.1/SUR2B channel hyperpolarizes the membrane resulting in the closure of VDCCs and producing vasodilation. Heart muscle cells mainly contain the Kir6.2/SUR2A isoform, which is suggested to be involved in cardioprotection in ischemic conditions. During ischemia, the opening of cardiac  $K_{ATP}$  channels shortens the action potential duration and limits the harmful  $Ca^{++}$  influx (Nichols and Lederer, 1991; Nichols et al., 1991; Noma, 1983). The molecular identities of  $K_{ATP}$  channels in the neurons are less known.  $K_{ATP}$  channels have been found in “glucose sensitive neurons” of hypothalamus and in pro-opiomelanocortin (POMC) neurons. Like in the  $\beta$  cells, the elevated ATP closes the  $K_{ATP}$  channel, depolarizes the membranes, and increases the excitation of neurons. Kir6.2 is suggested to be the major isoform of  $K_{ATP}$  channel in certain neurons (e.g., rat dorsal vagal neurons, hypothalamus neurons) (Karschin et al., 1998; Miki et al., 2001) while Kir6.1 and SUR1 have been detected in another study on glucose-receptive neurons of rat ventromedial hypothalamus (Lee et al., 1999). The opening of neuronal  $K_{ATP}$  channel may protect neurons under pathological conditions like ischemia and hypoxia by suppressing the neuronal activity (Soundarapandian et al., 2007b). The detailed mechanisms underlying the inhibition of the neural activity by glucose through  $K_{ATP}$  channel are still under investigation.

Besides the plasma membrane expression,  $K_{ATP}$  channels are also found in the mitochondria. Surprisingly, the molecular identity of these  $K_{ATP}$  channel is still elusive and the channel conductance is not consistent with any of the known  $K_{ATP}$  channels (Soundarapandian et al., 2007b).

### **2.3.4 Biophysical properties of $K_{ATP}$ channels**

Physiological and pharmacological analysis has characterized distinct properties of  $K_{ATP}$  channels in different tissues. Native  $K_{ATP}$  channels display quite different unitary conductance, for example, currents with ~70-90 pS were detected in cardiac muscle; ~55-75 pS in skeletal muscle and ~50-90 pS in pancreatic  $\beta$ -cells. In vascular smooth muscle, although a large current has been reported initially, the major evidence supports a channel conductance ~ 15-25 pS (Kajioka et al., 1991; Kamouchi and Kitamura, 1994). NDP is indispensable for the activity of this channel (Beech et al., 1993).

Cloned Kir6.2 containing channels have a conductance of 80 pS while in Kir6.1 containing channel, it is ~30 pS. Kir6.1/SUR2B channels resemble native vascular  $K_{ATP}$  channels, having a unitary conductance of ~35 pS and low basal activity in the absence of ATP (Quayle et al., 1997; Yamada et al., 1997).

### **2.3.5 $K_{ATP}$ channel opener and inhibitor**

Unlike other Kir channels where there is no specific channel opener and blocker,  $K_{ATP}$  channel can be inhibited and activated rather specifically due to the presence of SUR subunit. The  $K_{ATP}$  channel inhibitors and openers are classified into two groups of therapeutic compounds known as sulfonylureas and KCOs, respectively (Hibino et al., 2010).

Sulfonylureas include glibenclamide, tolbutamide, acetohexamide, etc. These drugs were initially used to treat typhoid fever but were unexpectedly found to cause hypoglycemia, which later was used to alleviate the hyperglycemia seen in diabetes. Currently these drugs are mainly used for the treatment of type 2 diabetes because of their specific inhibitory effects on the  $K_{ATP}$  channels of  $\beta$ -cells (Hibino et al., 2010). In this dissertation, we used glibenclamide as the specific  $K_{ATP}$  channel inhibitor, because it is a reliable and potent inhibitor for  $K_{ATP}$  channel in a variety of preparations.

The KCO is another group of drugs composing of pinacidil, diazoxide, nicorandil, cromakalim, etc. They are used outside of USA to reduce blood pressure and control hypertension. Nicorandil, one of the KCOs, is used for the treatment of angina pectoris in Japan. Interestingly, different  $K_{ATP}$  channels respond to KCOs differently, which is attributed to the different SUR isoforms. Research found that  $\beta$ -cell type  $K_{ATP}$  channels (with SUR1 isoform) respond to diazoxide effectively and to pinacidil weakly. These channels are insensitive to cromakalim or nicorandil. On the other hand,  $K_{ATP}$  channels expressed in cardiac muscle (SUR2A isoform) are sensitive to pinacidil, cromakalim and nicorandil with little sensitivity to diazoxide. The smooth muscle isoform of  $K_{ATP}$  channels are sensitive to all of these KCOs because of the SUR2B isoform of the SUR subunit (Hibino et al., 2010). Among these KCO, we chose to use pinacidil to activate the  $K_{ATP}$  channels to achieve large currents and to obtain better experimental resolution.

### **2.3.6 The regulation of $K_{ATP}$ channel by metabolites**

Kir6.2 containing channels are highly sensitive to metabolic states especially the ATP/ADP ratio. ATP in its free acid form is a strong channel inhibitor (Ashcroft and Kakei, 1989). Each Kir6.2 subunit has one ATP binding site so four ATP molecules can bind to a single

K<sub>ATP</sub> channel. However, the binding of one ATP is sufficient to cause channel closure (Markworth et al., 2000). In the presence of Mg<sup>++</sup>, however, ATP maintains the channel activity through PtdIns(4,5)P<sub>2</sub>, which is generated by ATP-dependent lipid kinase from phosphatidylinositol (Huang et al., 1998). NDPs including ADP are also essential for the channel activity. They increase the channel activity independently of ATP. This activation effect of NDP requires the presence of Mg<sup>++</sup> and the binding site of NDP is located in the SUR subunit (Matsuo et al., 2000). Therefore, a K<sub>ATP</sub> channel has two distinct sites (a stimulatory NDP-binding site and an inhibitory ATP-binding site) to sense the metabolic state.

The Kir6.1-containing channels, including the vascular Kir6.1/SUR2B channel, have low open probability in physiological conditions and only respond to the ATP modestly. Therefore, the Kir6.1-containing channels are often known as K<sub>NDP</sub> channels. Not only is the vascular K<sub>ATP</sub> channel regulation attributed to cellular metabolism directly, but also their activity relies largely on other factors that are related to metabolic states. For example, low pH, which occurs during the metabolic stress, has been shown to drastically activate vascular K<sub>ATP</sub> channel (Santa et al., 2003; Wang et al., 2003). In addition, these channels are targeted by a number of neurotransmitter or circulating hormones (discussed in below).

Each Kir6.2 subunit has a binding site for one molecule of ATP, and the binding of one ATP to one subunit is enough to cause channel closure. Therefore, in the physiological conditions when ATP concentration is in millimolar range, the K<sub>ATP</sub> channel is supposed to remain closed. However, K<sub>ATP</sub> channel activity recorded on native  $\beta$ -cells is significantly higher. It is likely that K<sub>ATP</sub> channel integrates the stimulatory inputs from MgADP, phospholipids, adenylate kinase, long-chain acyl CoA as well as the action of other molecules including



neurotransmitters or hormones to reduce ATP-mediated channel inhibition in these cells (Remedi and Koster, 2010).

### **2.3.7 Regulation of $K_{ATP}$ channel by vasodilators and vasoconstrictors**

Since vascular  $K_{ATP}$  channels are not a typical “ATP-sensitive”  $K^+$  channel, vasodilators and vasoconstrictors have been suggested to be the major players affecting the activity of these channels. The potent endogenous vasodilator calcitonin gene-related peptide (CGRP), found in perivascular nerves, activates  $K_{ATP}$  channels of coronary arteries or mesenteric arteries (Miyoshi and Nakaya, 1995; Nelson et al., 1990). Since the receptors of CGRP are G-protein coupled receptors, the activation of  $K_{ATP}$  channel is probably mediated through the PKA signaling pathway. More recent studies in our lab indicate that isoproterenol (Isop) activates the vascular  $K_{ATP}$  channel and leads to vasodilation (Shi et al., 2007b; Yang et al., 2008; Zhang et al., 2010b). This effect relies on the activation of PKA signaling pathway modulating the SUR2B subunit (Shi et al., 2008a). In contrast, the vasoconstrictor arginine vasopressin (AVP) inhibits the vascular  $K_{ATP}$  channel mainly through PKC signaling pathway modulating Kir6.1 subunit (Shi et al., 2007a). Nitric oxide (NO) is also an important vasodilator, however, its effect has only been observed in Kir6.2 but not in Kir6.1 containing channels. In this dissertation, we tested the effect of another important peptide hormone, vasoactive intestinal peptide (VIP), on the  $K_{ATP}$  channels.

### **2.3.8 $K_{ATP}$ channel knockout mice**

$K_{ATP}$  channels are made up of pore-forming Kir6.x (6.1 or 6.2) and regulatory SURx (SUR1 and SUR2). Mice lacking any of the four essential genes of  $K_{ATP}$  channels have been generated and studied. Kir6.2 and SUR1 knockout mice have impaired glucose homeostasis (Miki et al., 1998; Shiota et al., 2002). Mice without either Kir6.2 or SUR1 have transient

neonatal hypoglycemia (Seghers et al., 2000). In stress conditions, Kir6.2 knockout mice have an abnormal prolongation of the action potential of the heart with strong  $\text{Ca}^{++}$  overload, which leads to heart failure and death (Yamada et al., 2006). Kir6.2 is also expressed in the brain, and the Kir6.2 knockout mice have been observed to be susceptible to seizure after brief hypoxia (Yamada et al., 2001). The knockout of Kir6.1 or SUR2 causes the coronary artery impairments (Chutkow et al., 2001; Miki et al., 2002). Specifically, the Kir6.1 knockout mice develop Prinzmetal angina and have a very high risk of sudden death. They also have spontaneous elevation of S-T segments followed by an atrioventricular block. These syndromes indicate that the problem of sudden death is likely due to myocardial ischemia in these knockout mice.

### **2.3.9 $\text{K}_{\text{ATP}}$ channelopathies**

The mutation of  $\text{K}_{\text{ATP}}$  channels are identified in many diseases and more importantly, the causality link has been established between the mutations of  $\text{K}_{\text{ATP}}$  channels and these diseases. One of the most popular diseases caused by  $\text{K}_{\text{ATP}}$  channel mutation is neonatal diabetes mellitus. This disease is a rare disorder that refers to diabetes diagnosed within 6 months of birth (Flanagan et al., 2006). The mutations of  $\text{K}_{\text{ATP}}$  channels contribute to roughly 40% of the diagnosed cases of permanent neonatal diabetes. In physiological conditions, the glucose is up-taken into the  $\beta$  cells via the GLUT2 transporter. Glucose is then metabolized, resulting in an elevation of intracellular ATP concentration. The binding of ATP to the Kir6.2 subunit of the  $\text{K}_{\text{ATP}}$  channel leads to the channel closure. The closure of  $\text{K}_{\text{ATP}}$  channel causes membrane depolarization, triggers the activation of VACC, induces  $\text{Ca}^{++}$  influx and eventually insulin secretion. The gain-of-function mutations of either Kir6.2 or SUR1 subunits result in a  $\text{K}_{\text{ATP}}$  channel with a higher intrinsic open probability than the WT, making the channel less sensitive to the ATP-mediated inhibition. The impaired channel closure disrupts the insulin secretion,

resulting in diabetes. While most of the gain-of-function  $K_{ATP}$  channel mutations lead to diabetes, a small number of the patients develop a more severe syndrome called severe developmental delay and epilepsy syndrome (DEND) in addition to neonatal diabetes. This becomes the case when the mutations occur at a very critical site affecting the channel gating or other essential features. Strikingly, it has been reported that ~90% of DEND syndrome patients bear Kir6.2 mutations (Edghill et al., 2010).

The loss-of-function mutations of either Kir6.2 (KCNJ11) or SUR1 (ABCC9) of the  $K_{ATP}$  channel are one of the most popular causes of persistent hyperinsulinemic hypoglycemia of infancy (PHHI), characterized by uncontrolled secretion of insulin. The hypoglycemia associated with this disease irreversibly damages the brain. Mutations of  $K_{ATP}$  channels (~200 distinct mutations) account for ~70% of all PHHI cases. These mutations affect several different aspects of  $K_{ATP}$  channels: one group of mutations affect the membrane expression of  $K_{ATP}$  channels (Ashcroft, 2005), another group of mutations cause the inactivity of the  $K_{ATP}$  channels (Ashcroft, 2005), and the third group disrupts the channel sensitivity to nucleotides (Remedi and Koster, 2010).

Besides the dysfunction of  $K_{ATP}$  channel in  $\beta$ -cells, recent studies indicate that frame-shift and missense mutation of SUR2A genes in patients lead to dilated cardiomyopathy (Bienengraeber et al., 2004). Unlike other diseases caused by mutations of  $K_{ATP}$  channel, the underlying mechanism and the causality link in the SUR2A mutations and frame-shift have not been established.

## 2.4 Protein Posttranslational Modification: PKA

### 2.4.1 Definition of the posttranslational modification

Protein posttranslational modifications (PTMs) refer to the later steps in protein biosynthesis and can be used to describe any protein modifications that occur after the maturation of the proteins. In general, these modifications involve chemical reactions to form covalent bonds between proteins and small biological molecules (except the formation of disulfide bond between two cysteine residues). PTMs can be divided into two groups. The first group of PTMs occurs constantly and routinely after the protein biosynthesis and is an essential step towards the maturation of the proteins. For example, after the biosynthesis, insulin is cut twice after the formation of disulfide bonds, followed by removal of a propeptide for maturation. The second group of PTM occurs only in response to the specific stimuli, changing the behavior of the proteins or altering the function of the proteins (e.g. protein phosphorylation). This group of PTMs often involves the addition of functional groups (Zhang et al., 2010a). Many PTMs are facilitated by specific enzymes while others are through non-enzymatic reactions (e.g. oxidization reaction). The first group of PTMs does not fit in the scope of our study therefore we will mainly focus on the second group of PTMs.

Over hundreds different PTMs have been identified, while more than a dozen of them have been observed in *in vivo* conditions (Zhang et al., 2010a). The biologically relevant PTMs involving the addition of functional groups includes: acetylation, methylation, glycosylation, S-nitrosylation, S-glutathionylation, phosphorylation and many more. PTMs also involve the addition of peptides including different ubiquitins (Bhave and Gereau, 2004; Rougier et al., 2010).

### **2.4.2 Protein phosphorylation as a PTM in physiological condition**

Protein phosphorylation is by far the most well understood PTM, in which a phosphate ( $\text{PO}_4$ ) group is added to the proteins (Ubersax and Ferrell, 2007). Phosphorylation is found in a large number of proteins involved in virtually all cellular processes. Phosphorylation of a protein can be achieved by the action of protein kinases like PKA, PKC and PKG and many others. Protein phosphorylation occurs on specific residues (serine, threonine and tyrosine). The protein kinases often recognize certain motifs but many phosphorylation sites reported do not fully fall into the classic consensus amino acid sequences. In this dissertation, we studied how VIP activates the vascular  $\text{K}_{\text{ATP}}$  channel through the PKA signaling pathway.

### **2.4.3 Neurotransmitters**

Receptor-mediated intracellular signaling often leads to protein phosphorylation, especially the metabotropic receptors. The binding of the ligands to the receptor activates the G-protein and further triggers the intracellular signaling cascade, for example, the PKA signaling system. Another type of receptors is ionotropic receptors, which acts as a ligand-gated ion channel, directly converting the signals from neurotransmitters to the gating of ion channels. Their signaling may lead to protein phosphorylation as well, through other signaling molecules such as  $\text{Ca}^{++}$ .

Many neurotransmitters, including glutamate (Glu), serotonin (5-HT), acetylcholine (ACh), gamma-aminobutyric acid (GABA), adenosine triphosphate (ATP) have both ionotropic as well as metabotropic receptors. On the contrary, others (dopamine, norepinephrine, epinephrine, vasoactive intestinal peptide) are known to be associated only with metabotropic receptors. Glycine is among the few neurotransmitters that are only found to be associated with ionotropic receptors.

Ionotropic receptors are further divided depending on the type of ions passing through. For example, NMDA and AMPA receptors (bind to Glu); Nicotinic acetylcholine receptor, (bind to Ach); 5-HT<sub>3</sub> (bind to 5-HT); P2X (bind to ATP), are a group of ionotropic receptors that conduct cations. Glycine receptors (bind to Gly); GABA<sub>A</sub> receptors (bind to GABA) are a group of ionotropic receptors that conduct anionic (Cl<sup>-</sup>) currents.

#### **2.4.4 GPCR signaling pathway**

GPCRs are a large group of receptors that are coupled to a variety of signaling pathways including PKA and PKC. The binding of ligand (neurotransmitters, hormones, etc) to the specific GPCR initiates the cascade. The activation of GPCRs through receptor-ligand interaction leads to the conformational change of the GPCRs, and activates the G protein. Several different G-proteins exist that are associated with different GPCRs. The G proteins are divided into G $\alpha$  and G $\beta\gamma$ . G $\alpha_s$  and G $\alpha_{i/o}$  proteins are involved in cAMP dependent pathways. G $\alpha_s$  activates the adenylate cyclase (AC) and leads to the conversion of cytosolic adenosine triphosphate (ATP) to cAMP, whereas G $\alpha_{i/o}$  inhibits the AC activity and leads to the decrease in cAMP level. Since the activity of Protein kinase A relies on the levels of cAMP, GPCR associated G $\alpha_s$  and G $\alpha_{i/o}$  trigger the downstream signaling pathways mainly through the regulation of PKA activity. G $\alpha_{q/11}$ , on the other hand, activates phospholipase C- $\beta$ , which in turn cleaves the membrane bound phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) and make inositol (1,4,5) triphosphate (IP<sub>3</sub>) and diacylglycerol (DAG). IP<sub>3</sub> binds to IP<sub>3</sub> receptors of the endoplasmic reticulum (ER) and cause the release of Ca<sup>++</sup> from the ER into the cytoplasm. DAG that remains bound to the plasma membrane activates several membrane localized forms of PKC. Many isoforms of PKC can also be activated by an increase of intracellular Ca<sup>++</sup> level. These two pathways converge together to activate the PKC signaling pathway. G $\alpha_{12/13}$  is coupled to RhoGEFs (p115-RhoGEF, PDZ-

RhoGEF and LARG), activating small GTPase. Other G proteins exist including G $\beta\gamma$ . These G proteins mainly target the ion channels including the GIRK (G-protein coupled inwardly rectifying K<sup>+</sup> channels) and P/Q- or N-type voltage gated Ca<sup>++</sup> channels.

#### **2.4.5 PKA consensus sequence**

Protein kinase A preferentially phosphorylates the serine and threonine residues in a protein. The consensus sequence for PKA has been shown to be (R-R-X-S/T- $\Phi$ , where S/T is a serine/threonine phosphorylation site and  $\Phi$  represents a hydrophobic amino acid residue). The S/T phosphorylation site is referred to as P, the first residue C-terminus to the P is P-1 while the first residue N-terminus of P is referred as P+1, and so on for the remaining residues on both sides of P. The crystal structure of PKA bound with PKI (non-phosphorylatable substrate mimetic) has been resolved, which provides structural explanation for this consensus sequence (Ubersax and Ferrell, 2007).

Several key structural elements in PKA that limit the choice for potential substrates have been identified based on the charge and hydrophobic interactions. Two Glutamic acid (Glu) residues in PKA protein form anionic binding sites for the P-2 and P-3 positions of the substrate. A hydrophobic pocket serves as a high affinity site for a hydrophobic residue in the P+1 position. The consensus sequence of the substrate for PKA fits well into the local structure and provides the primary specificity for the substrate and kinase interaction. Other residues in the substrate also contribute to the binding of PKA and its substrates. These residues sometimes are farther from the active site so that this could be a key factor to explain why some phosphorylation sites do not fully fall into the classic motifs R-R-X-S/T- $\Phi$  (Ubersax and Ferrell, 2007).

## **2.5 PTM: S-glutathionylation**

### **2.5.1 Oxidative stress overview**

Oxidative stress is the condition in which the excessive ROS production overwhelms the cellular anti-oxidant system (Madamanchi et al., 2005). Oxidative stress is also referred to as a condition that can cause pathological PTMs. Several endogenous ROS exist including superoxide ( $O_2^{\cdot-}$ ), hydroxyl radicals ( $\cdot OH$ ), hydrogen peroxide ( $H_2O_2$ ), and ozone ( $O_3$ ) (Powers and Jackson, 2008).

Oxidative stress is known to cause protein dysfunction by the action of excessive ROS, affecting mainly the cysteine residues of proteins. In the cardiovascular system, oxidative stress plays a leading role in hypertension, arteriosclerosis and other cardiovascular diseases by affecting the key molecules involving in physiological regulation of blood vessel tone. Many key molecules, including vascular  $K_{ATP}$  channels, have been found to be targeted by ROS. In the coronary arteries of diabetic patients (Miura et al., 2003) and cerebral arterioles from the rat (Erdos et al., 2004; Ross and Armstead, 2003), the inhibition of  $K_{ATP}$  channel by oxidative stress or ROS is also seen (Ichinari et al., 1996; Krippeit-Drews et al., 1999). Therefore, fundamental questions remain open as to which isoforms of  $K_{ATP}$  channel are targeted by oxidative stress, and what molecular mechanisms underlie the  $K_{ATP}$  channel modulation.

### **2.5.2 Reactive oxygen/nitrogen species**

Superoxide is an intermediate in many biochemical reactions. Compared with other free radicals, it has a relatively long half-life and can diffuse away from its original source to modify proteins in a distinct location. It is membrane impermeable and relatively less reactive than  $\cdot OH$ . Superoxide rapidly reacts with NO or iron-sulfur containing proteins forming  $ONOO^-$  or  $\cdot OH$ . Dismutation of  $O_2^{\cdot-}$  by superoxide dismutase (SOD) is a major source of endogenous  $H_2O_2$ .



H<sub>2</sub>O<sub>2</sub> is the most stable endogenous ROS. It can pass through plasma membranes across water channels and chloride channels. Compared with other ROS, H<sub>2</sub>O<sub>2</sub> is a relatively weak oxidizing agent. Catalase (Cat) is the primary enzyme reducing the endogenous H<sub>2</sub>O<sub>2</sub> to water and O<sub>2</sub> (Madamanchi et al., 2005). In this dissertation, we mainly used H<sub>2</sub>O<sub>2</sub>, an endogenously existing ROS, to induce oxidative stress, and tested how the oxidative stress affects the function of vascular K<sub>ATP</sub> channels.

Hydroxyl Radicals is the most reactive ROS and the strongest oxidizing agent.  $\cdot\text{OH}$  has a very short half-life and is membrane impermeable. It is the most damaging ROS that exist endogenously. However, due to its high reactivity and extreme short half-life, the reliable methods to measure the  $\cdot\text{OH}$  are limited and indirect, making it difficult to attribute directly the damaging effect of ROS to  $\cdot\text{OH}$  (Madamanchi et al., 2005).

### **2.5.3 S-glutathionylation**

S-glutathionylation is a post-translational modification mechanism of proteins at the cysteine residues by adding a glutathione (GSH) moiety (Dalle-Donne et al., 2008). Protein S-glutathionylation is facilitated by the oxidative stress when excessive ROS are present and GSH is available. S-glutathionylation allows the regulation of a large number of cellular processes by modulating proteins to alter their functions. Before S-glutathionylation mechanism is identified, the general idea for oxidative stress or ROS/RNS mediated protein modification was thought to be mediated through the formation of disulfide bonds or sulfenic acid intermediates like ( $-\text{SOH}$ ,  $-\text{SO}_2\text{H}$  or  $-\text{SO}_3\text{H}$  acids). However, since GSH is abundant within the cells, GSH is likely to serve as a major electron donor to ROS and make S-glutathionylation a dominate mechanism underlying the protein modification in oxidative stress.

#### **2.5.4 Sulphydryl or thiol groups (–SH) as reactive center**

Thiol group is susceptible to oxidative modification. In biological system, it exists in cysteine residues of proteins or in small molecules like glutathione and dihydrolipoic acid. Upon redox reaction, the thiol group is modified in response to the stimuli and local environment and thus affects the function of the proteins having such accessible thiol groups. Depending upon the nature of stimulation, different modification may occur. (1) ROS may cause the formation of disulfide bond between two cysteine residues within a protein or between two disparate proteins. (2) In the presence of certain amount of GSH, ROS stimulation may lead to S-glutathionylation. (3) In the presence of NO, ROS stimulation may cause S-nitrosylation. (4) Oxidation of cysteine residues may also result in the sequential formation of cysteine radicals ( $P-S^{\cdot}$ ), followed by sulphenic (PS-OH), sulphinic (PS-O<sub>2</sub>H) and eventually sulphonic (PS-O<sub>3</sub>H) acids. The last two reactions are irreversible. In this dissertation, we tested if S-glutathionylation is the molecular mechanism underlying the channel inhibition under oxidative stress (Dalle-Donne et al., 2008).

#### **2.5.5 Glutathione**

Glutathione exists in virtually all cells in the millimolar concentration range. It is the major non-protein thiol compounds that act as an inherent antioxidant, and work together with oxidized glutathione (GSSG) as intracellular redox buffer (Dalle-Donne et al., 2008). GSH is a tripeptide, containing a cysteine, a glutamate and glycine through unusual peptide bonds, in which the carboxyl group of the glutamate side-chain is linked to the amine group of cysteine. The carboxyl group of cysteine is linked to the amine group of glycine in a conventional manner. In mammalian cells, the concentration of GSH ranges from 1~5 mM depending on the cell types. Most of them exist in the form of GSH and a small portion of them exist as GSSG. In

physiological conditions, the GSH: GSSG ratio is around 50:1 to 100:1 but this ratio can change drastically in pathological conditions e.g., oxidative stress. ROS tends to accept electrons so that GSH is an antioxidant by serving as an electron donor. In this process, glutathione is turned into GSSG.

### **2.5.6 Antioxidant and deglutathionylation enzyme**

Protein deglutathionylation is catalyzed by specific enzymes. Glutaredoxin (Grx) is suggested to be the major deglutathionylation enzyme in mammalian cells (Meyer et al., 2009; Mieyal et al., 2008). The mammalian cytosolic form of Grx (Grx1) is exclusively selective for protein-SSG compared with other forms of disulfides (e.g. S-S disulfide bond, S-nitrosylation, etc.). It is very effective in reducing protein-SSG, thus is considered as a specific deglutathionylating enzyme. Moreover, in Grx1 knockout mice, no deglutathionylating activity was detected (Meyer et al., 2009), further supporting its critical role. Therefore, we used Grx1 to test whether S-glutathionylation mediated channel inhibition is reversible.

Besides Grx1, other enzymes have been implied in deglutathionylation or serving in antioxidant defense system, including thioredoxin (Trx2), peroxiredoxins (Prx3/5), thioredoxin reductase (Trxr2), glutathione reductase (GR) and etc. However, the specificity and the specific substrates of these enzymes are still not well understood (Lin and Beal, 2006).

### **2.5.7 Specificity of protein S-glutathionylation**

The specificity of protein S-glutathionylation has not been fully understood and is still under wide debate (Dalle-Donne et al., 2008). In general, it is believed that not all cysteine residues are equally susceptible to S-glutathionylation upon the same stimuli (e.g. GSSG). However, no consensus motif has been found relating to S-glutathionylation. S-

glutathionylation is mediated by oxidants and so far no strong evidence indicating enzymes can facilitate this process.

Several different contributing factors conferring specificity have been pointed out. Firstly, the accessibility of the thiol group of a given cysteine residue in the protein three-dimensional structure is the primary factor determining if the thiol of this cysteine could undergo S-glutathionylation. The reactivity of the cysteine residues is another determining factor depending on its adjacent residues or microenvironment. It has been suggested that if the cysteine is surrounded with positive charged residues, it is more reactive because a P-S<sup>+</sup> may form through the interaction of the thiol group with the charged residues. Another words, a cationic environment can make the –SH group particularly susceptible to S-glutathionylation. Thirdly, the helix-dipole effect has been indicated to contribute to the reactivity of the cysteine residues, which could lower the pKa of cysteine residues. Moreover, cysteine residues form hydrogen bonds with charged residues e.g. Ser or His, selenocysteine residues or cysteines bound with metal ions, e.g. Mg<sup>++</sup>, Ca<sup>++</sup> or Zn<sup>++</sup> are also readily to be S-glutathionylated (Dalle-Donne et al., 2008).

### **2.5.8 Structure impact of protein S-glutathionylation**

Protein S-glutathionylation results in the incorporation of a GSH moiety to the protein cysteine residue. This incorporation depends on the location of the cysteine and may affect the protein structure directly. Consequently, the charged group of GSH and the charged groups around the cysteine residue may interact with each other to affect the protein structure. In either case, the S-glutathionylation mediated structural changes may lead to alteration of protein functions. To understand how the S-glutathionylation structurally affects the protein function, the availability of the crystal structure of the target protein is essential. Structural insights have been

gained through the modeling of pore forming Kir6.1 subunit of K<sub>ATP</sub> channel using the crystal structure of bacterial and chicken Kir channel as templates in this dissertation.

## **2.5.9 S-glutathionylation of ion channels**

### **S-glutathionylation of RyR**

Ryanodine receptor (RyR) is the first ion channel found to be S-glutathionylated, as well as S-nitrosylated (Sun et al., 2001b, 2003). Expressed on the endoplasmic reticulum (ER), the RyR is responsible for the Ca<sup>++</sup> release from ER to cytosol. The RyR protein has ~100 cysteine residues and is highly sensitive to oxidation. Both S-glutathionylation and S-nitrosylation could occur on RyR channels: It has been reported that GSSG and H<sub>2</sub>O<sub>2</sub> enhance the channel activity by reducing the Mg<sup>++</sup> mediated inhibitory effect while NO donor NOR-3 enhances the Ca<sup>++</sup> mediated activation of channel kinetics. GSNO, which can cause both S-glutathionylation and S-nitrosylation, produces both effects. (Aracena et al., 2003). Further study has demonstrated that certain cysteine residues (Cys1040 and Cys1303) are exclusively S-nitrosylated, and some (Cys1593 and cys3193) are selectively S-glutathionylated, while others can undergo both reactions (Aracena-Parks et al., 2006). However, these studies open interesting questions: How can S-nitrosylation and S-glutathionylation selectively occur on certain cysteine residues? Do these modulations occur in the similar manner in the in vivo condition? How does S-nitrosylation compete with S-glutathionylation for the same residue? What are the determinants for the reaction? What is the physiological significance for the presence of such complicated modulation systems?

### **S-glutathionylation of CFTR**

Cystic fibrosis transmembrane conductance regulator (CFTR) is a chloride channel belonging to the ABC transporter family. CFTR regulates the salt and water transportation across

epithelial membranes in the lung and gut. Mutations of CFTR gene can lead to cystic fibrosis and congenital bilateral absence of vas deferens. During inflammation, ROS produced target the epithelial cells and modulate the plasma membrane-bound CFTR. Indeed, Wang et. al., found several oxidized forms of glutathione including GSSG (10~20 mM), and nitrosylated glutathione/S-nitrosoglutathione (GSNO, 50~200  $\mu$ M) that can inhibit the channel activity markedly in excised patch recordings. Moreover, the combined application of glutathione and diamide (5~100  $\mu$ M, equimolar), an S-glutathionylation induction method, can also cause marked channel inhibition. These reactions could be rescued by the reducing agent dithiothreitol (DTT, 20 mM) and deglutathionylation enzyme glutaredoxin (Grx, 4  $\mu$ M). In addition, biochemical experiments further demonstrate that diamide (100  $\mu$ M) facilitates the incorporation of biotin-GSH (125  $\mu$ M) to CFTR protein, which could be pulled down by streptavidin and be further detected by western blot. Using mutagenesis, Cys344 located in the NBD2 domain is identified as the primary site for S-glutathionylation. This modulation potentially affects the nucleotide binding and disrupts the ATP-dependent channel opening. The data demonstrate that CFTR is S-glutathionylated in their experimental conditions. However, this study does not rule out the possibility that the naturally occurring GSNO can also cause S-nitrosylation to inhibit the channel activity. Moreover, additional studies using naturally occurring ROS including  $\text{H}_2\text{O}_2$  or XO/X system that are of physiological relevance in addition to diamide will be needed (Wang et al., 2005).

### **S-glutathionylation of CRAC channel**

$\text{Ca}^{++}$  release-activated  $\text{Ca}^{++}$  (CRAC) channels belong to store-operated channels, which play important roles in a variety of signaling cascades of non-excitable cells, including T lymphocytes and mast cells. Recent studies have identified Orai1 ( $\text{Ca}^{++}$  release-activated  $\text{Ca}^{++}$

modulator) as the pore-forming subunit of CRAC channel while Stromal-interacting molecule (STIM) as the endoplasmic reticulum (ER)  $\text{Ca}^{++}$  sensor. Upon  $\text{Ca}^{++}$  store depletion, STIM1 which is located in ER undergoes oligomerization and redistributed to the location that is within 10-25 nm of the plasma membrane (Penna et al., 2008; Prakriya et al., 2006). With such a short distance, STIM1 on the ER is able to bind to Orai1 directly and causes its activation, leading to  $\text{Ca}^{++}$  influx (Park et al., 2009a). In oxidative stress, the impairment of  $\text{Ca}^{++}$  signaling has been observed but the underlying mechanism is not clear (Bogeski et al., 2010). Hawkins et. al., reported that oxidative stress, induced by Lipopolysaccharide (LPS) (1.5  $\mu\text{g/ml}$ ),  $\text{H}_2\text{O}_2$  (100  $\mu\text{M}$ ) or buthionine sulfoximine (BSO, 200  $\mu\text{M}$ ,  $\gamma$ -glutamyl synthetase, the rate-limiting enzyme in GSH synthesis, inhibitor) alter the  $\text{Ca}^{++}$  signaling in B-lymphocytes by oxidizing the glutathione and through the modification of the CRAC channels (Hawkins et al., 2010). They further identified that the ER-resident STIM1 is S-glutathionylated by pull-down experiment. STIM1 is immunoprecipitated with  $\alpha$ - STIM1 antibodies and then probed for S-glutathionylation with a peptide antibody against GSH. A conservative cysteine residue (Cys56) is further identified as the target of S-glutathionylation. The addition of GSH to STIM1 is further confirmed by mass spectra. The S-glutathionylation of STIM1 results in a constitutively activated CRAC channel. This paper provides good evidence indicating that S-glutathionylation occurs on the Cys56 of STIM1. However, since the truncated STIM1 (N-terminal region) is used for the experiment, it is not known if other cysteine residues in STIM may also be S-glutathionylated. Moreover, since this region is located within the ER lumen, the concentration of GSH and the ratio between GSH/GSSG is not known, call for further experiments. This paper also fails to rule out the involvement of Orai S-glutathionylation, which could also be possible.

### **S-glutathionylation of channels for $\text{Ca}^{++}$ homeostasis**

Intracellular  $\text{Ca}^{++}$  concentration and  $\text{Ca}^{++}$  oscillations play important roles in endothelial cells, including the regulation of vascular permeability, inflammation, etc. The disruption of  $\text{Ca}^{++}$  homeostasis has been associated with oxidative stress, whereas the molecules that are directly targeted by ROS are not clear. Lock et. al., demonstrate that diamide, a thiol-oxidizing agent, could increase the intracellular  $\text{Ca}^{++}$  concentration and oscillations (Lock et al., 2011). The authors further demonstrate that this modulation is related to glutathione by using N,N'-bis(2-chloroethyl)-N-nitrosourea (BCNU), an inhibitor of glutathione reductase and L-buthionine-sulfoximine (BSO), which could inhibit the  $\gamma$ -glutamylcysteine synthetase. They further identified that  $\text{IP}_3$  receptors and plasmalemmal  $\text{Ca}^{++}$ -ATPase (PMCA) pump are the molecules targeted by ROS and tested the hypothesis that the modulation of these two molecules is through S-glutathionylation using pull-down experiments with BioGEE. Based on their data, they conclude that S-glutathionylation occurs on  $\text{IP}_3$  receptors and PMCA pumps to enhance the activity of the former but decrease the activity of the later. This study provides the first pieces of evidence about the S-glutathionylation of  $\text{IP}_3$  and PMCA pump but further studies are definitely needed to dissect the detailed modulation mechanism, especially of the cysteine residues. Although diamide has been used extensively in the experimental condition as a strong oxidizing agent, but it is not an endogenous substance. It would be interesting to see if the similar responses described in this report could be obtained using more rigid methods and stimuli that are more physiologically relevant (e.g.,  $\text{H}_2\text{O}_2$ ,  $\text{ONOO}^-$ , etc.) to induce S-glutathionylation (Lock et al., 2011).



## 2.6 Other forms of ion channel PTMs

### 2.6.1 Ion channel S-nitrosylation

S-nitrosylation is referred to as the addition of an NO to the thiol moiety of a cysteine residue. S-nitrosylated Cysteine residues are often found in an acid-base motif with an acidic amino acid (D/E) in the +1 amino acid (AA) next to the cysteine residue being critical (Gonzalez et al., 2009).

Specificity of this regulation is partially contributed by the spatial co-localization of the target proteins with nitric oxide synthases (NOSs), the enzymes that generate NO in vivo. No enzyme that can facilitate the formation of protein S-nitrosylation has been found. Enzymes like S-nitrosogluthathione reductase (GSNOR) and thioredoxins (Trx1 and Trx2) have been shown to mediate the de-nitrosylation. Several methods including biotin switch protocol (Forrester et al., 2007; Jaffrey and Snyder, 2001), photolysis chemiluminescence (Xu et al., 1998) and antibodies against S-nitrosocysteine moiety (Aracena et al., 2005) have been developed to detect the S-nitrosylation.

A large number of ion channels have been identified to undergo S-nitrosylation. RyR is a well demonstrated example for S-nitrosylation. The RyR located on the SR membrane mediates  $\text{Ca}^{++}$  release of SR in response to  $\text{Ca}^{++}$  influx through plasma membrane  $\text{Ca}^{++}$  channel. RyR has ~100 cysteine residues and about half of them are reactive. Some of them are found to be S-nitrosylated and others are S-glutathionylated, respectively. The S-nitrosylation occurs on Cys3635 of RyR1, which is located within a hydrophobic motif (Sun et al., 2001a). The different effects of NO on RyR are attributed largely to the specific types of NO donors, the concentration of the NO donor and the channel redox state (Gonzalez et al., 2008).

S-nitrosylation occurs on voltage-gated  $\text{Na}^+$  channel  $\text{Na}_v1.5$  (SCN5A), a channel responsible for fast depolarization in C-type DRG neurons. S-nitrosylation of this channel is responsible for the persistence of  $\text{Na}_v1.5$  current in cardiac myocytes and inhibition of the currents in sensory neurons (Renganathan et al., 2002). Voltage-gated  $\text{K}^+$  channels, which determine the resting membrane potential, have been found to be S-nitrosylated. In KCNQ1 channel, S-nitrosylation has been found to occur at Cys445, located within a consensus motif (Asada et al., 2009).  $\text{K}_v1.5$ , which generates the ultra-rapid component of the delayed rectifier ( $I_{\text{kur}}$ ), is inhibited by S-nitrosylation. Based on the modeling studies, it is suggested that the S-nitrosylation of two cysteine residues located in the voltage-sensor region of the channel affect the channel conformational change (Nunez et al., 2006).

Transient receptor potential (TRP) channel family is a group of cation channels that play important roles in a variety of cellular processes by mediating the  $\text{Ca}^{++}$  signaling (Clapham, 2003). S-nitrosylation modulation has been tested on all the members of TRPC branch (TRPC1-TRPC7) and interestingly, only TRPC3 has been shown to respond to the NO donor SNAP (300  $\mu\text{M}$ ) and  $\text{H}_2\text{O}_2$  (1 mM) significantly. Further test using reactive disulfides, mutagenesis as well as biochemical experiments demonstrate that the NO mediated TRPC channel modulation is through S-nitrosylation of TRPC5 at a conserved site Cys558. Structural modeling shows that this site is located at the pore-forming helix, and the S-nitrosylation of which cause the bend of the helix leading to channel opening (Yoshida et al., 2006).

It is worth noting that S-nitrosylation and S-glutathionylation shares lots of common features: they are both facilitated by oxidants; they all target the cysteine residues and attach a moiety to the cysteine residues. Therefore, in the experimental condition, reactive disulfides can be used to mimic both S-nitrosylation and S-glutathionylation. Moreover, studies have shown

that for a given protein, S-nitrosylation and S-glutathionylation both could occur depending on the specific cysteine residues and the redox state of the proteins (Aracena-Parks et al., 2006). Standard protocols and testing strategies should be carefully evaluated and established to guide potential future studies to further distinguish between the S-nitrosylation and S-glutathionylation.

### **2.6.2 Ion channel S-palmitoylation**

Protein S-palmitoylation refers to the formation of a reversible thioester linkage of a 16 carbon palmitate lipid to an intracellular cysteine residue. This modification has been shown to control the maturation, trafficking and the regulation of ligand- and voltage- gated ion channels. It has also been shown to affect the channel modulation by other signaling molecules (Shipston, 2011). This modulation is reversible. The enzymes that facilitate this modulation are called palmitoyl acyltransferases and zDHHC (zinc finger- and DHHC domain- containing proteins) while the de-palmitoylation is achieved through the enzyme (palmitoyl thioesterases). The major effect of palmitoylation is to increase the hydrophobicity of the target protein, which may affect the function of ion channels in a variety of aspect including their surface expression and intrinsic activity.

For example, palmitoylation has been shown to affect the trafficking and surface expression of ion channels including Nav1.2 and Kv1.6, NMDA receptors, BK channels and many more. On the other hand, palmitoylation can also modulate the channel activity, like the intrinsic voltage sensitivity of Kv1.1 channel (Gubitosi-Klug et al., 2005), gating of epithelial Na<sup>+</sup> channel (Mueller et al., 2010) and voltage-dependent inactivation of N-type Ca<sup>++</sup> channels (Stephens et al., 2000).

Besides, there is a potential crosstalk or interaction between palmitoylation and other PTMs. It has been further suggested that palmitoylation inhibits the channel phosphorylation by PKC through steric hindrance and promotes channel phosphorylation in some other cases.

Although no “consensus” motif for protein palmitoylation has been identified, some factors critical for the cysteine palmitoylation (Fukata and Fukata, 2010) have been found. The local concentration of fatty acyl-CoA close to the reaction site, the local environment of the target cysteine and the accessibility of the cysteine, whether there are basic or hydrophobic residues around it can determine whether palmitoylation is likely to occur or not (Belanger et al., 2001).

### **2.6.3 Ion channel SUMOylation and Ubiquitylation**

Ubiquitylation is mainly involved in the degradation pathways through internalization and targeting of membrane proteins to the lysosome. Ubiquitin is a small protein of 7kDa, activated by an enzyme called E1 and then transferred by E2 to E3. The interaction of E3 ubiquitin ligases with their substrates would add an ubiquitin to the lysine residues of the target proteins. Targeted proteins can be attached with either mono- or multiple- ubiquitin proteins (Rougier et al., 2010).

The SUMOylation pathway, however, is involved in many cellular functions. It often associated with transcriptional activity. SUMO stands for Small Ubiquitin-related MOdifier. SUMOylation usually occurs on lysine residues that fit into the consensus motif of  $\psi$ -K-X-E/D ( $\psi$  represents aliphatic acid and X represent any amino acid). De-SUMOylation requires a group of enzymes called SENP (SUMO/sentrin-specific protease)

Many ion channels have been shown to undergo Ubiquitylation. For example,  $\text{Na}_v 1.5$ , a major voltage-gated  $\text{Na}^+$  channel in cardiac muscle, is ubiquitylated. This modification depends

on the ubiquitin ligase nedd4-2, a member of the Nedd4 (neuronal precursor cell developmentally downregulated 4) family. Nedd4-2 enhances the internalization rate of Na<sub>v</sub> 1.5 channel (Rougier et al., 2005; van Bemmelen et al., 2004). However, how this regulation affects the cellular function is still not clear. KCNQ1 channel mediated the delayed rectifier K<sup>+</sup> current I<sub>ks</sub>, playing a key role in the action potential repolarization phase. The delayed rectifier K<sup>+</sup> current (I<sub>ks</sub> current) density is shown to be reduced by many Nedd4 family members without altering its biophysical properties (Jespersen et al., 2007). Human ERG1 channel encodes the  $\alpha$ -subunit of the cardiac K<sup>+</sup> current delayed rectifier I<sub>kr</sub>. The trafficking of hERG1 is strongly associated with the ubiquitylation.

Channel SUMOylation has been initially identified in the K2P1 channel. K2P1 is a background leaking K<sup>+</sup> channel, with two pore-forming P loops in each subunit. Two subunits form a functional channel. These channels play important roles in setting the resting membrane potential of the cells and control the cell excitability. K2P1 gene does not produce currents in experimental conditions, although its mRNA has been well detected. It is later shown that SUMOylation of the K2P1 channel is the reason for its silencing and de-SUMOylation of this channel using SENP-1 would increase the channel activity (Rajan et al., 2005). However, these findings are later challenged by other groups (Feliciangeli et al., 2007) and the debates are still going on with new evidence emerging (Feliciangeli et al., 2010; Plant et al., 2010). Moreover, SUMOylation has been found on K<sub>v</sub>2.1 channel, where this modification inhibits the current of K<sub>v</sub>2.1 to affect the cellular excitability and action potential (AP) (Dai et al., 2009). K<sub>v</sub>1.5 channel is critical for atrial AP repolarization and the sumoylation of this channel induces the alteration of the steady-state inactivation of this channel but does not cause any change in current density (Benson et al., 2007).

## 2.6.4 Ion channel O-GlcNAcylation

O-linked  $\beta$ -N-acetyl-glucosamine (O-GlcNAc) glycosylation (O-GlcNAcylation) is a PTM which differs from the “classical” N-linked glycosylation. It occurs on cytoplasmic proteins, membrane proteins at the cytosolic side and also nuclear proteins (Hurtado-guerrero et al., 2008). It modifies serine and threonine residues by O-linked attachment of a single  $\beta$ -N-acetyl-glucosamine moiety. UDP-GlcNAc, an end product of the hexosamine biosynthetic pathway, is the substrate for O-GlcNAcylation. O-GlcNAcylation is reversible reaction, facilitated by the enzyme O- $\beta$ -N-acetylglucosaminyltransferase (OGT) and removed by O- $\beta$ -N-acetylglucosaminidase (OGA). O-GlcNAcylation is highly associated with pathological conditions and also serves as a sensor of cellular stress. The substrate of O-GlcNAcylation, UDP-GlcNAc, increases significantly in hyperglycemia, hyperlipidemia and diabetes conditions. Stress conditions like oxidative stress, nutrient stress and thermal stress can induce O-GlcNAcylation.

Since O-GlcNAcylation occurs on serine and threonine residues similar to protein phosphorylation, interplay between O-GlcNAcylation and phosphorylation has been suggested (Wang et al., 2008). As a result, O-GlcNAcylation mediated protein modification could either block or promote the phosphorylation on the same site or site at a distant location (Hu et al., 2010), affecting the protein functions.

Ion channels are critical in maintaining the vascular tones and cardiac contractile. As vascular and cardiac dysfunctions have been linked with hyperglycemia and diabetes, (Chatham, 2010; Clark, 2003; Lima et al., 2008b; Lima et al., 2009), it is likely that ion channels could be the target of O-GlcNAcylation during these pathological conditions (Lima et al., 2008a). Although more than 100 proteins have been found to be O-GlcNAcylated, the study of O-

GlcNAcylation on ion channels are still in the state of infancy. To date, only one ion channel for intracellular  $\text{Ca}^{++}$  release (InsP<sub>3</sub> receptor I) has been found to be GlcNAcylated (Rengifo et al., 2007). O-GlcNAcylation decreases the activity of InsP<sub>3</sub> receptor type I but increases the activity of InsP<sub>3</sub> receptor type 3. InsP<sub>3</sub> receptor type 2 is also found to be O-GlcNAcylated but no functional consequence has been observed (Bimboese et al., 2011). Functional assays of channel activities have been obscured due to the low levels of activity of the recombinant OGT enzyme (Rengifo et al., 2007). With the rapid progress in O-GlcNAcylation research, better reagents and protocols would surely facilitate the understanding of ion channel O-GlcNAcylation in the near future.

## **2.7 The production and action of ROS/RNS**

### **2.7.1 Mitochondrial enzymes**

Mitochondria are the metabolic center of the cells and have numerous redox enzymes for transferring electrons to oxygen and generate reactive oxygen species. A number of enzymes have been found to generate ROS in mitochondria, which include the enzymes involved in tricarboxylic acid (TCA) cycle, and the electron-transport chain (ETC) complexes, I, II, and III (Lin and Beal, 2006). However, under normal physiological conditions, the ROS are rapidly cleared up by the extensive endogenous anti-oxidant enzymes and molecules. Therefore, both the membrane bound and soluble proteins and other molecules are protected from oxidative stress (Lin and Beal, 2006). Mitochondria have a powerful antioxidant defense system which includes both non-enzymatic and enzymatic components. The former is made of GSH,  $\alpha$ -tocopherol ( $\alpha$ TCP), cytochrome C and coenzyme Q10, and the later is made of manganese superoxide dismutase (MnSOD), catalase (Cat), glutaredoxin (Grx2), thioredoxin (Trx2), and many more. The detailed functions of these enzymes can be seen in published reviews (Andreyev et al.,

2005). Among these enzymes, it is clear that Glutaredoxin (Grx2) catalyzes Trx-disulfide oxidoreduction reactions. It can reduce both protein disulfide and mixed disulfides with GSH, while thioredoxins efficiently reduce only protein disulfides (Andreyev et al., 2005).

### **2.7.2 NADPH oxidase**

The NADPH oxidase (NOX) family NADPH oxidase generates ROS (mainly  $O_2^{\cdot-}$  and  $H_2O_2$ ) through electron transfer from NADPH to molecular oxygen. Superoxide generation by NADPH oxidase was originally believed to be a unique property of immune cells. The most well-known NOX is NOX2/gp91phox, firstly demonstrated in professional phagocytes. Many more NOX family members have lately been identified that make the current NOX a big family. Like NOX2, the main functions of these proteins are to generate superoxide by transporting electrons. Since the NOX family has been identified in virtually all cell types, it is clear that the effect of NADPH oxidases is not limited to host defense system. Rather, it is shown to be involved in post-translational processing of proteins, cellular signaling, and regulation of gene expression (Bedard and Krause, 2007). NOX activity is regulated by  $Ca^{++}$ , which is affected by the membrane excitability of the cells (Sumimoto, 2008).

NOX has been found to regulate ion channels. For example, NOX has been found to regulate the activity of TASK channel. NOX4 co-localizes with TASK and the overexpressing of NOX4 and TASK channel in the HEK293 cells reduces the current amplitudes of TASK channel at both +60 mV and -60 mV voltage conditions. Moreover, the overexpression of NOX4 but not NOX2 enhances the  $O_2$  sensitivity of TASK-1. On the other hand, knockdown of the endogenous NOX4 expression or the presence of NADPH oxidase inhibitors blunts the  $O_2$ -dependent regulation of TASK-1 (Lee et al., 2006). These data indicate that by simply overexpression, NOX may execute its function. However, this paper does not address whether it is the ROS



produced by NOX or some other mechanism that affect the sensitivity of TASK-1 to oxygen. Angiotensin II, which is coupled with the PKC signaling pathway and is a potent vasoconstrictor, has been suggested to be the most potent inducer of NOX (George and Struthers, 2009). Therefore, it would be interesting to see if the Angiotensin II treatment could enhance the phenomenon observed in their studies.

### **2.7.3 Xanthine oxidase**

XO was initially found to be involved in metabolizing hypoxanthine, purine degradation, and xanthine to uric acid with the generation of superoxide. It was later found to be involved in ischemia-reperfusion injury, endothelial dysfunction, hypertension and heart failure (Berry and Hare, 2004). XO is abundantly expressed in liver and small intestine of mammals. However, the expression of XO is also found in bovine heart, rat heart, and gland, capillary endothelial cells (Berry and Hare, 2004). Superoxide radicals produced from XO readily react with endothelial NO to form  $\text{ONOO}^-$ , thereby reducing the bioavailability of NO and impairs vasorelaxation. NO and superoxide react at an extremely fast rate so even when SOD is present, it would fail to eliminate superoxide (Cai and Harrison, 2000).

Xanthine (50  $\mu\text{M}$ ) plus xanthine oxidase (50  $\text{mU ml}^{-1}$ ) are the common ways to generate superoxide (Bielefeldt et al., 1997; Duprat et al., 2005). Other literature shows that XO (0.75  $\text{mU/ml}$ ) and hypoxanthine HX (125  $\mu\text{M}$ ) can not only raise  $\text{O}_2^{\cdot-}$  levels but also increase  $\text{H}_2\text{O}_2$  (Kelley et al., 2010). Since SOD decreases  $\text{O}_2^{\cdot-}$  and increases  $\text{H}_2\text{O}_2$  and catalase decreases  $\text{H}_2\text{O}_2$ , using SOD and catalase can provide additional information to further distinguish the involvement of  $\text{H}_2\text{O}_2$  (Juncos and Garvin, 2005).

Besides, other ROS could be generated as follows: Iron catalyzed Fenton reaction with superoxide generates hydroxyl radicals (Bedard and Krause, 2007). Tert-butyl hydroperoxide

(tBHP) and  $\text{H}_2\text{O}_2$  also produce hydroxyl radicals (Duprat et al., 2005). Rose Bengal can produce singlet oxygen ( $^1\Delta\text{gO}_2$ ) followed by photo-activation (Duprat et al., 2005).

#### **2.7.4 The combined effect of ROS and NO**

$\text{ONOO}^-$  is a strong and relatively stable oxidant species (Pacher et al., 2007).  $\text{ONOO}^-$  is able to pass the cell membrane to some extent through anion channels.  $\text{ONOO}^-$  is formed in tissue by the non-enzymatic interaction between  $\text{O}_2^{\cdot-}$  anion and NO (Liu et al., 2002). This reaction is the dominating reaction so that in the presence of ROS, the bioavailability of NO will drastically decrease, which leads to the impairment of vasodilatation as well as other vascular diseases. The formed  $\text{ONOO}^-$  is also a strong reactive species, which reacts with proteins, lipids, carbohydrates, and nucleic acids leading to the vascular dysfunction. In experimental condition, NO can be generated using SNP (10 nM) or 3-morpholiniosydnonimine (SIN-1, 1 mM) and superoxide can be made using X/XO system (xanthine, X, 0.1 mM and xanthine oxidase, XO, 0.01 U/ml) (Liu et al., 2002). The combination of these compounds thus would lead to the formation of  $\text{ONOO}^-$  (Beckman and Koppenol, 1996; Duprat et al., 2005).  $\text{ONOO}^-$  can be synthesized using another published method (Brzezinska et al., 2000).

#### **2.8 Introduction to this dissertation**

Evidence by the literature, we have designed a series of experiments in this dissertation. We found that an exposure of isolated mesenteric rings to  $\text{H}_2\text{O}_2$  impaired the  $\text{K}_{\text{ATP}}$  channel-mediated vascular dilation. In whole-cell recordings and inside-out patches, micromolar  $\text{H}_2\text{O}_2$  or diamide caused a strong inhibition of the vascular  $\text{K}_{\text{ATP}}$  channel (Kir6.1/SUR2B) in the presence, but not in the absence, of GSH, suggesting the involvement of S-glutathionylation. Systematic mutational analysis revealed three contributing cysteine residues (Cys43, Cys120 and Cys176) to

be important. Among them, Cys176 was prominent. Simulation modeling of Kir6.1 S-glutathionylation revealed that after incorporation to residue 176 the GSH moiety occupied a space between slide helix and two transmembrane helices, preventing the necessary conformational change of the M2 inner helix for channel gating, and retained the channel in its closed state. We further found that the channel was strongly augmented by VIP and the channel activation relied on PKA phosphorylation.

### 3. SIGNIFICANCE

Cardiovascular diseases (CVD) are a major challenge to modern medicine (Goldberg and Dansky, 2006; Grundy et al., 2002). A critical stage in the pathogenesis of the diseases is oxidative stress, a leading contributor to several other diseases including diabetes, inflammation and neurodegenerative diseases. Oxidative stress results from the over-production of reactive oxygen, carbonyl or nitrogen species that exceeds the cellular anti-oxidant defense systems. Although oxidative stress is widely accepted as the key contributor to the CVDs, non-selective anti-oxidants treatment fails to improve the outcome of patients with cardiovascular diseases in clinic trials. This suggests that oxidative stress should not be viewed as an isolated detrimental factor which can be simply managed by anti-oxidants. Rather, it involves a more complicated cascade including initiators, mediators and effectors. A deeper understanding of the downstream molecules that are involved in the sustaining of oxidative stress thus becomes critical. The investigation of how the reactive species production becomes a cascade may lead to a novel approach to control oxidative stress and improve the outcome of CVDs.

Previous studies have found that the production of reactive species is closely associated with membrane excitability (Schilling and Elliott, 1992; Takeya and Sumimoto, 2003; Trebak et al., 2010). The damaging effects of oxidative stress could be accelerated and amplified in the condition where the cells are hyper-excitable. Membrane hyper-excitability causes  $\text{Ca}^{++}$  influx, activation of NOX enzyme and more ROS production. Therefore, the functions of key molecules that regulate membrane excitability may be vital determinants of the extent of oxidative stress. Their dysfunction can produce persistent depolarization and hyper-excitability, leading to  $\text{Ca}^{++}$  overload, more reactive species production, and structural damages.

Vascular  $\text{K}_{\text{ATP}}$  channel is a key regulator of membrane excitability. The activation of

vascular  $K_{ATP}$  channel leads to hyperpolarization, reduction of the  $Ca^{++}$  influx and protection against  $Ca^{++}$  overload. In diabetic patients and laboratory animal models for diabetes and fluid percussion brain injury, the dysfunction of  $K_{ATP}$  channels is seen, and oxidative stress is subsequently developed followed by pathogenesis in multiple organs, especially the vasculature. Therefore, vascular  $K_{ATP}$  channel seems to be a central player in this pathological process, mediating or facilitating the ROS production. Hence, activation of the vascular  $K_{ATP}$  channel might be a potential approach to counteract the cellular hyper-excitability, prevent the ROS production cascade, and alleviate oxidative stress. VIP, a peptide hormone, is known to be protective against oxidative stress (Koh et al., 2009; Offen et al., 2000; Steingart et al., 2000; Yu et al., 2011). In P12 neuroblastoma cells and cerebellar granule neurons, VIP has a protective role against oxidative stress (Offen et al., 2000). It has been found that VIP protects the STZ-induced diabetes mice against oxidative stress and inflammation (Yu et al., 2011). In SMCs, which are also excitable cells, VIP is likely to protect the cells against oxidative stress as well, probably by activating the vascular  $K_{ATP}$  channels.

Considering the potential role of  $K_{ATP}$  channels in the link of the ROS production and membrane excitability, the demonstration of how the vascular  $K_{ATP}$  channel is modulated in oxidative stress is of critical importance. The understanding of the  $K_{ATP}$  channel modulation in oxidative stress may shed new insight into how membrane excitability is affected by oxidative stress, how the ROS production cascade occurs with hyper-excitability, what is the molecular basis of  $K_{ATP}$  channel modulation by different reactive species, and how the cascade can be interrupted by manipulating the  $K_{ATP}$  channel activity. The information gained in this study is not limited to cellular physiology but also have a profound impact on the design of therapeutic modalities for CVDs and other oxidative stress-associated diseases.

## **4. MATERIAL AND METHODS**

### **4.1 Chemicals and reagents.**

Unless stated, all reagents and chemicals used in this study were purchased from Sigma.  $\text{H}_2\text{O}_2$ , GSH were freshly made and used within 4 hrs. Other reagents were prepared in high-concentration stocks in double-distilled water or dimethyl sulfoxide (DMSO). In cases where DMSO was used, the final concentration of DMSO in solution was less than 0.1% and this concentration of DMSO did not have any detectable effect on the channel activity.

### **4.2 Mesenteric artery preparation and tension measurement.**

All animal experiments were performed in compliance with an approved protocol by the Institutional Animal Care and Use Committees (IACUC) at Georgia State University. Male Sprague-Dawley rats (200-250g body weight) were deeply anesthetized and sacrificed. Mesenteric arteries were dissected and the connective tissues were removed in PSS containing (in mM): NaCl 140, KCl 4.6,  $\text{CaCl}_2$  1.5  $\text{MgCl}_2$  1, glucose 10, HEPES 5, pH 7.3. The arteries were cut into small rings (2 mm in length) and transferred to ice-cold Krebs solution containing: NaCl 118.0,  $\text{NaHCO}_3$  25.0, KCl 3.6,  $\text{MgSO}_4$  1.2,  $\text{KH}_2\text{PO}_4$  1.2, glucose 11.0,  $\text{CaCl}_2$  2.5. The arterial ring was mounted on a force-electricity transducer (Model FT-302, iWorx/CBSciences, Inc. Dover, NH) for measurements of isometric force contraction in a 5-ml tissue bath filled with the air bubbled Krebs solution. All rings were pre-tested with phenylephrine (PE) to ensure the tissue vitality. When endothelium-free rings were needed, the rings were rubbed with a sanded polyethylene tubing. The endothelium-free rings were then tested for PE contraction followed by acetylcholine (Ach, 1  $\mu\text{M}$ ). The rings were considered to be endothelium-free if more than 90%

relaxation was eliminated. PE and ACh were then washed out, and the rings were allowed to equilibrate in the Krebs solution for another 30-60 min before the experiments (Yang et al., 2010; Yang et al., 2008).

#### **4.3 Expression of K<sub>ATP</sub> channel in HEK293 cells.**

All wild-type and mutant channels were expressed in HEK293 cells because these cells have minimum endogenous K<sub>ATP</sub> channels. The HEK293 cells were cultured in DMEM/F12 medium with 10% fetal bovine serum and Penicillin/streptomycin at 37 °C with 5% CO<sub>2</sub>. A eukaryotic expression vector pcDNA3.1 was used to express rat Kir6.1 (GenBank No. D42145) or mouse Kir6.2 (GenBank No. D50581) in the cells with SUR2B (GenBank No. D86038, mRNA isoform NM\_011511). A 35 mm petri dish of cells was transfected with 1 µg Kir6.1 or Kir6.2 and 3 µg SUR2B using Lipofectamine<sup>2000</sup> (Invitrogen Inc., Carlsbad, CA). To facilitate the identification of positively transfected cells, 0.4 µg green fluorescent protein (GFP) cDNA (pEGFP-N2, Clontech, Palo Alto, CA) was included in the cDNA mixture. One day after transfection, cells were disassociated with 0.25% trypsin, split and transferred to cover slips for further growth. Experiments were performed on the cells in cover slips during the following 12-48 hrs (Shi et al., 2007a; Shi et al., 2008a; Yang et al., 2010).

#### **4.4 Electrophysiology.**

Patch clamp experiments were carried out at room temperature as described previously (Shi et al., 2007a; Shi et al., 2010; Shi et al., 2008a; Shi et al., 2008b; Shi et al., 2007b; Yang et al., 2008). In brief, fire-polished patch pipettes with 2-5 MΩ resistance were made from 1.2 mm borosilicate glass capillaries. Whole-cell currents were recorded in single-cell voltage clamp with holding potential 0 mV and step to – 80 mV. The bath solution contained (in mM): KCl 10,

potassium gluconate 135, EGTA 5, glucose 5, and HEPES 10 (pH=7.4). The pipette was filled with a solution containing: KCl 10, potassium gluconate 133, EGTA 5, glucose 5, K<sub>2</sub>ATP 1, NaADP 0.5, MgCl<sub>2</sub> 1, and HEPES 10 (pH=7.4). To avoid nucleotide degradation, all intracellular solutions were freshly made and used within 4 hrs. All the recordings were made with the Axopatch 200B amplifier (Axon Instruments Inc., Foster City, CA). The data were low-pass filtered (2 kHz, Bessel 4-pole filter, -3 dB), and digitized (10 kHz, 16-bit resolution) with Clampex 9 (Axon Instruments Inc.). Macroscopic currents were recorded from giant inside-out patches, and single-channel currents were recorded from small inside-out patches with a constant single voltage of – 80 or – 60 mV. Symmetric high K<sup>+</sup> (145 mM in total) was used in both bath and pipette solution and K<sub>2</sub>ATP 1 mM and NaADP 0.5 mM was included in the bath solution to maintain the channel activity. Higher sampling rate (20 kHz) was used to digitize the currents recorded from inside-out patch. Data was analyzed using Clampfit 9 (Axon Instruments Inc.).

#### **4.5 Immunocytochemistry.**

Immunocytochemistry was performed on the HEK293 cells with or without Kir6.1/SUR2B transfection. Two hours before the experiments, the culture medium was replaced with fresh one. Biotinylated glutathione ethyl ester (BioGEE, 250  $\mu$ M, Invitrogen) was added to the medium and incubated for 1 hr followed by H<sub>2</sub>O<sub>2</sub> (750  $\mu$ M) challenge for 15 min. The medium was then discarded and the cells were washed 3 times with PBS (containing 0.3% Triton X-100) to remove the excessive free BioGEE that was not conjugated with proteins. Cells were then fixed with 4% paraformaldehyde for 30 min followed by 3 times of washing using PBS. Dylight-488 conjugated streptavidin was diluted in 1:1000 in PBS and added to the cells for 1 hr incubation at room temperature. After three washes, the cells were examined under regular or confocal microscopes. For double staining, the cells were further incubated with rabbit primary antibody



against Kir6.1 for 2 hrs followed by 3 washes. Dylight-594 conjugated goat anti-rabbit secondary antibody (1:1000, Jackson ImmunoResearch) was used to visualize the Kir6.1 staining. Experiments were repeated three times (Yang et al., 2010).

#### **4.6 Streptavidin pull-down assay and western blot.**

HEK293 cells expressing Kir6.1/SUR2B channels and the A10 smooth muscle cell line were used for the experiment. A10 cells were cultured in DMEM with 10% fetal bovine serum (FBS) at 37 °C in humidified atmosphere with 5% CO<sub>2</sub>. BioGEE and H<sub>2</sub>O<sub>2</sub> treatments were performed as described in *Immunochimistry*. Cells were then washed once and lyzed using RIPA buffer (Sigma). Samples were run on 10% SDS-polyacrylamide non-reducing gel and then transferred to a nitrocellulose membrane (Bio-Rad). Rabbit primary antibodies against Kir6.1 (1:500, Sigma) and secondary antibodies conjugated with alkaline phosphatase were used in the Western blot (1:10,000, Jackson ImmunoResearch). Signals were visualized by SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

Biotin-GSH conjugated proteins were pulled down using streptavidin-dynabeads according to the instruction of the provider (Invitrogen). Briefly, the beads were washed three times before the immobilization. Samples were then mixed with beads and incubated at room temperature with gentle rotation for 30 min. A magnet was used to separate the biotinylated molecules-beads complex with other unlabeled proteins. Supernatant containing unlabeled proteins was discarded and the pellet was resuspended followed by three washes. The biotinylated molecules-beads complex were then resuspended in the loading buffer with 0.1% SDS and boiled so that the glutathionylated proteins were released into solvent for further analysis. Experiments on HEK293 cells transfected with Kir6.1/SUR2B channel were repeated

four times and experiments on A10 cells were repeated five times (Yang et al., 2011; Yang et al., 2010).

#### **4.7 Mutagenesis and chimeras construction.**

Kir6.x channels were divided into 3 regions (N-terminus, core domain containing two transmembrane domains and the pore loop, and C- terminus). For Kir6.1, N terminus, core, and C terminus was referred to as residues 1-71, 72-186 and 187-424, respectively. For Kir6.2, corresponding domain was 1-70, 71-176, and 177-390, respectively. Kir6.1-Kir6.2 chimeras were created by overlap extension using PCR reaction using *pfu* DNA polymerase (Stratagene, La Jolla, CA). WT Kir6.1 was called 111. If the N- terminus was replaced by the corresponding regions from Kir6.2, we called it 211. Similarly, if the C-terminus of Kir6.1 was constructed to Kir6.2, we designated the chimerical channel 221. All the other possible combinations were also created and were named after this naming system. Site-directed mutagenesis kits (Stratagene) were used to introduce mutations. In these experiments, two oligonucleotide primers (30-35 bp) will be synthesized, each of which contains the same mutation and anneals to the same sequence on opposite strands of the cDNA. *Pfu* DNA polymerase was used to extend and incorporate the mutagenic primers leading to two nicked circular strands. *DpnI* was used to digest the nonmutated parental cDNA and the circular, nicked dsDNA was transformed to XL1-Blue supercompetent cells. The correct constructs and mutations were confirmed by sequencing (Shi et al., 2008a; Yang et al., 2011).

#### **4.8 Structural modeling.**

The native Kir6.1 structure was generated by I-TASSER protein structure prediction (Wu et al., 2007; Zhang, 2007, 2008) based on the most recent crystal structure of eukaryotic Kir2.2

(PDB entry: 3jyc) (Tao et al., 2009) and bacterial KirBac1.1 (PDB entry: 1p7b), KirBac3.1 (PDB entry: 1xl4) and prokaryotic Kir3.1 channel chimera (PDB entry: 2qks) as the templates (Kuo et al., 2003; Nishida et al., 2007). The Kir6.1 open state structure was generated using the KirBac1.1 open state model provided by Dr. Venien-Bryan (Kuo et al., 2005). The native Kir6.1 structural model was further modified by incorporating the GSH molecule onto the thiol group of Cys176. The potential locations and orientations of GSH in Kir6.1 structural model were determined using energy minimization by AMMP (Harrison and Weber, 1994) based on the interaction of charged groups of GSH with charged amino acid groups of Kir6.1 proteins. The one with lowest binding energy between GSH and the Kir6.1 protein was chosen for further analysis and presentation. Distances between molecules were measured with PyMol. The sequence alignment was generated by ClustalW2. Structural images were generated with PyMol molecular graphics system (Yang et al., 2011).

#### **4.9 Acute dissociation of mesenteric vascular smooth cells.**

Single vascular smooth cells were prepared with two-step enzyme digestions. Main branch of mesenteric arteries were obtained as mentioned above. After clearance of connective tissue, 1-2 mm small segments were cut and placed in 5ml solution containing (in mM): NaCl 140, KCl 5.4, MgCl<sub>2</sub> 1, CaCl<sub>2</sub> 0.1, HEPES 10 and D-glucose 10 for 10 min in room temperature. The tissues were then placed in 1ml of the same solution with 20 U of papain (Worthington, New Jersey), 1.25 mg DTT and 1% fetal bovine serum. After 25min digestion at 35°C, the tissues were washed and incubated with 440 U collagenase (CLS II, Worthington), 1.25 mg trypsin inhibitor (Sigma) and 1% fetal bovine serum for 10 min. After thorough washes, the tissues were triturated with a fire-polished Pasteur pipette to yield single cells. The dissociated smooth muscle cells were placed in a petri dish and allowed to attach to the dish

surface before recordings. Patch clamp experiments were carried out in the cells that show clear smooth muscle morphology, and had no sign of swelling and shrinkage (Shi et al., 2007a; Shi et al., 2008a; Yang et al., 2008).

#### **4.10 Data analysis.**

Data were presented as means  $\pm$  standard error (s.e.). Differences were tested by Student's *t* test or analysis of variance (ANOVA). Significant differences were accepted when *P* was  $< 0.05$ .

## 5. RESULT 1: OXIDATIVE STRESS INHIBITS VASCULAR K<sub>ATP</sub> CHANNELS BY S-GLUTATHIONYLATION

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Contribution disclosure: Yang Yang performed patch clamp experiments, biochemical assays (immunochemistry, western blot and pull down experiment), cell culture, data analysis, and wrote the paper draft. Ningren Cui, Weiwei Shi and Zhongying Wu assisted with patch clamp study. Dr. Chun Jiang and Yang Yang designed the research.

## 5.1 Abstract:

The  $K_{ATP}$  channel is an important player in vascular tone regulation. Its opening and closure lead to vasodilation and vasoconstriction, respectively. Such functions may be disrupted in oxidative stress seen in a variety of cardiovascular diseases, while the underlying mechanism remains unclear. Here, we demonstrated that S-glutathionylation was a modulation mechanism underlying oxidant-mediated vascular  $K_{ATP}$  channel regulation. An exposure of isolated mesenteric rings to  $H_2O_2$  impaired the  $K_{ATP}$  channel-mediated vascular dilation. In whole-cell recordings and inside-out patches,  $H_2O_2$  or diamide caused a strong inhibition of the vascular  $K_{ATP}$  channel (Kir6.1/SUR2B) in the presence, but not in the absence, of GSH. Similar channel inhibition was seen with GSSG and thiol-modulating reagents. The oxidant-mediated channel inhibition was reversed by the reducing agent DTT and the specific deglutathionylation reagent glutaredoxin-1 (Grx1). Consistent with S-glutathionylation, pull-down assays with BioGEE showed incorporation of GSH to the Kir6.1 subunit in the presence of  $H_2O_2$ . These results suggest that S-glutathionylation is an important mechanism for the vascular  $K_{ATP}$  channel modulation in oxidative stress.

## 5.2 Introduction:

$K_{ATP}$  channels are regulated by intracellular ATP/ADP and couple intermediary metabolic states to membrane excitability. The activity of these channels is low under physiological conditions and drastically rises during metabolic stress (Miki and Seino, 2005). The  $K_{ATP}$  channels are expressed in almost all tissues. In SMCs, activation of  $K_{ATP}$  channels by several vasodilators reduces SMC membrane excitability, leading to vasorelaxation (Shi et al., 2007b; Yang et al., 2008). Activity of the channels is inhibited by vasoconstrictors (Shi et al., 2007a), resulting in depolarization of the SMCs and vasoconstriction. Such a common vascular regulator is thus targeted by a variety of cellular events in physiological and pathological conditions (Miki and Seino, 2005; Teramoto, 2006).

One important cellular event is oxidative stress that is known to play an important role in the development and maintenance of several cardiovascular diseases, such as hypertension, atherosclerosis, and diabetic vascular complications (Brownlee, 2001; Madamanchi et al., 2005). During oxidative stress, excessive reactive oxygen species (ROS), such as superoxide, hydroxyl radicals, and  $H_2O_2$ , are overly produced causing vascular dysfunction and structural damages (Ardanaz and Pagano, 2006; Gutterman et al., 2005). Previous studies indeed have shown that  $O_2^-$  suppresses  $K_{ATP}$  channels and blunts the pial arterial dilation responses (Armstead, 1999). In diabetic patients, in whom oxidative stress is evident,  $K_{ATP}$  channel function is disrupted, leading to impaired vasodilation responses (Miura et al., 2003). In insulin-resistant rats, the  $K_{ATP}$  channel-dependent vasodilation is also impaired, which is likely to be mediated by ROS (Erdos et al., 2004). Although the dysfunction of  $K_{ATP}$  channels in oxidative stress has been documented, the mechanism underlying channel modulation remains unknown (Gutterman et al., 2005; Weintraub, 2003).

ROS can modulate proteins by intra- and intermolecular thiol oxidation (Moran et al., 2001), which may be the underlying cause for the modulation of vascular  $K_{ATP}$  channels in oxidative stress. To test this hypothesis, we performed studies using a combined molecular biology, electrophysiology, and biochemistry approach. Our results showed that the Kir6.1/SUR2B channel, the major isoform of vascular  $K_{ATP}$  channels, was inhibited by micromolar concentrations of  $H_2O_2$  as well as several other oxidants via S-glutathionylation.



## 5.3 Results

### 5.3.1 H<sub>2</sub>O<sub>2</sub> impaired pinacidil-induced vasodilation in isolated mesenteric rings.

In order to define conditions whereby oxidative stress disrupts vascular K<sub>ATP</sub> channel function, we examined the vasodilating effects of the K<sub>ATP</sub> channel opener in the presence and absence of H<sub>2</sub>O<sub>2</sub>. Experiments were conducted on endothelium-intact (EI) and endothelium-denuded (ED) mesenteric rings. Vasoconstriction was first produced with 30 mM K<sup>+</sup> in ED rings. This was followed by treatments with increasing concentrations of pinacidil, a specific K<sub>ATP</sub> channel opener and a strong vasodilator (Fig. 5-1A). The vascular tones were measured with a force-electricity transducer. A pre-treatment of the rings with H<sub>2</sub>O<sub>2</sub> (300, 600 μM) impaired the pinacidil-induced vasodilation in both ED and EI rings. In ED rings, the IC<sub>50</sub> concentration of pinacidil for vasorelaxation was raised by 5–18 folds with the 300 μM and 600 μM H<sub>2</sub>O<sub>2</sub> treatments, respectively (Fig. 5-1A, B). Similar results were obtained in EI rings (Fig. 5-1C). Taken together, our data indicate that the function of vascular K<sub>ATP</sub> channels is disrupted in oxidative stress.

### 5.3.2 H<sub>2</sub>O<sub>2</sub> inhibited Kir6.1/SUR2B channel activity in the presence of GSH.

The Kir6.1/SUR2B channel is the major isoform of vascular K<sub>ATP</sub> channels (Cao et al., 2002; Teramoto, 2006; Tricarico et al., 2006). Thus, we studied its modulation by expressing the Kir6.1/SUR2B channel in HEK293 cells. In whole-cell voltage clamp, the baseline Kir6.1/SUR2B currents were small, and no obvious effect on the currents was observed when H<sub>2</sub>O<sub>2</sub> was applied. After the currents were activated by pinacidil, however, the Kir6.1/SUR2B channel was dose-dependently inhibited by H<sub>2</sub>O<sub>2</sub> with an IC<sub>50</sub> of 1.53 mM (Fig. 5-2A, E, F).

The results of whole-cell recordings may be affected by washout or inadequate controls of cytosolic soluble factors that can be potentially involved in the channel modulation, such as

endogenous GSH and GSSG. Therefore, further studies were performed in excised patches. In giant inside-out patches, millimolar concentrations of  $\text{H}_2\text{O}_2$  were required to inhibit the Kir6.1/SUR2B channel in the absence of cytosolic soluble components (Fig. 5-2B, E, F). Strikingly, the administration of small amount of GSH drastically enhanced the channel sensitivity to  $\text{H}_2\text{O}_2$ . In the presence of 100  $\mu\text{M}$  of GSH,  $\text{H}_2\text{O}_2$  as low as 10  $\mu\text{M}$  began to inhibit channel activity, and clear concentration dependence was seen with an  $\text{IC}_{50}$  of 25  $\mu\text{M}$  (Fig. 5-2C, E, F). The effect of  $\text{H}_2\text{O}_2$  was subsequently studied in the presence of 2 mM GSH and 40  $\mu\text{M}$  GSSG, a ratio that is close to the physiological concentrations of GSH/GSSG in the cytosol (Waypa et al., 2006). Under this condition,  $\text{H}_2\text{O}_2$  also potently inhibited the channel with an  $\text{IC}_{50}$  of 20  $\mu\text{M}$  (Fig. 5-2D-F).

Consistent with these observations in inside-out patches, a supplement of GSH/GSSG (2 mM and 40  $\mu\text{M}$ , respectively) to the pipette solution significantly enhanced the channel sensitivity to  $\text{H}_2\text{O}_2$  in whole-cell recordings. With 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$ , the whole-cell currents were inhibited by  $36.2 \pm 8.8\%$  ( $n = 5$ ) instead of by  $8.0 \pm 5.1\%$  ( $n = 4$ ) without these substances in the pipette ( $p < 0.05$ , Fig. 5-2G). In comparison, the same concentration of  $\text{H}_2\text{O}_2$  (100  $\mu\text{M}$ ) inhibited the channel by  $>80\%$  in inside-out patches in the presence of GSH or GSH/GSSG (Fig. 5-2G). The difference in the  $\text{H}_2\text{O}_2$  sensitivity between inside-out patches and whole-cell recordings may be due to the diffusion kinetics across plasma membranes. Using the pseudo-first-order reaction analysis described by Tang et al. in their study on  $\text{H}_2\text{O}_2$ -mediated BK channel inhibition (Tang et al., 2004), we calculated the pseudo first order constant ( $724 \text{ M}^{-1} \text{ min}^{-1}$ ) on the basis of the average Kir6.1/SUR2B channel inhibition (36.2%) by 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$  in a period of  $<5$  min. With the rate constant, our further calculation showed that a 50% inhibition of the channel was achieved by  $\sim 23 \mu\text{M}$   $\text{H}_2\text{O}_2$  in  $\sim 30$  min. The requirement of GSH for  $\text{H}_2\text{O}_2$  to produce its channel

inhibition effect indicates that GSH-mediated protein modifications of the Kir6.1/SUR2B channel, such as S-glutathionylation, are likely to occur when  $\text{H}_2\text{O}_2$  is produced as an intermediary metabolite or a product of oxidative stress.

To understand the biophysical mechanisms underlying Kir6.1/SUR2B modulation, we analyzed single channel properties recorded in regular inside-out patches in the presence of 2 mM GSH and 40  $\mu\text{M}$  GSSG. We found that the channel open-state probability was progressively suppressed with increased  $\text{H}_2\text{O}_2$  concentrations while the unitary conductance remained unchanged (Fig. 5-2D).

### **5.3.3 GSH-dependent modulation by other oxidants.**

Diamide, an oxidant that produces intra- and intermolecular disulfide bonds, is known to cause S-glutathionylation in the presence of GSH (Kil et al., 2008; Wang et al., 2005). In giant inside-out patches, we found that the joint application of diamide and GSH drastically inhibited channel activity within 2–8 min, regardless of the order of application, i.e.,  $78.4 \pm 5.1\%$  inhibition with GSH first and  $87.9 \pm 8.7\%$  inhibition with diamide first (Fig. 5-3A, B, E). No statistical difference was found between the experiments ( $P > 0.05$ ,  $n = 12$ ). In contrast, neither GSH (Fig. 5-3C, E) nor diamide alone (Fig. 5-3D, E) resulted in such channel inhibition over an extended time period (8~14 min), suggesting that the channel inhibition is unlikely to be a result of the formation of disulfide bonds within a protein or between proteins.

GSSG is another S-glutathionylation inducer (Kil et al., 2008; Wang et al., 2005). In giant inside-out patches, we found that the application of GSSG inhibited the Kir6.1/SUR2B channel in a concentration-dependent manner (Fig. 5-4A, B). Evident channel inhibition was seen with 0.5 mM GSSG, and stronger inhibition was produced by higher concentrations (Fig. 5-

4A, B). Together, these data strongly suggest that S-glutathionylation appears to occur in the Kir6.1/SUR2B channel during oxidative stress leading to the inhibition of the channel activity.

#### **5.3.4 Thiol oxidants inhibited the Kir6.1/SUR2B channel.**

Several reactive 2-pyridinedisulfides (2-PDSs; Fig. 5-5A, C) are known to target the free sulfhydryl groups of cysteine residues forming thiol moieties, a protein modulation mechanism that resembles S-glutathionylation (Yoshida et al., 2006). Thus, we further tested the thiol oxidation of the Kir6.1/SUR2B channel using several 2-PDSs. In whole-cell recording, the bath application of 2, 2'-dithiodipyridine (2-DTP; 50  $\mu$ M), a membrane permeable 2-PDS, inhibited the Kir6.1/SUR2B currents almost completely (by  $95.8 \pm 1.1\%$ ;  $n = 5$ ; Fig. 5-5D, E). Similar results were obtained with another membrane permeable 2-PDS, 2, 2'-dithiobis-5-nitropyridine (DTNP; 50  $\mu$ M), which inhibited the currents by  $88.8 \pm 2.7\%$  ( $n = 5$ ; Fig. 5-5B, E). These observations are also consistent with S-glutathionylation.

#### **5.3.5 Reversal of the $K_{ATP}$ channel inhibition by deglutathionylation reagents.**

After pinacidil-activated currents were strongly inhibited by the oxidants, washout with addition of pinacidil (10  $\mu$ M) had only a modest effect on channel activity ( $10.2 \pm 2.6\%$  of the original pinacidil-induced currents;  $n = 4$ ; Fig. 5-2C). Increasing the pinacidil concentration to 100  $\mu$ M had no additional effect ( $3.9 \pm 0.6\%$  of the original pinacidil induced-current,  $n = 3$ ; Fig. 5-3A). However, application of the reducing agent DTT (5 mM) reversed the GSSG-mediated (5 mM) current inhibition by  $36.1 \pm 4.7\%$  ( $P < 0.001$ ;  $n = 4$ ; Fig. 5-4C, E). Similarly, the 2-PDS mediated channel inhibition was partially reversed by DTT by  $34.9 \pm 10.3\%$  ( $n = 5$ ; Fig. 5-5D, E).

An exposure of the cell to the specific deglutathionylation reagent glutaredoxin-1 (Grx1; 4  $\mu$ M) immediately reversed current inhibition by  $27.8 \pm 10.5\%$  ( $P < 0.05$ ;  $n = 4$ ). After washout of both GSSG and Grx1, the pinacidil-induced currents were resumed to  $72.0 \pm 7.1\%$  of its original level ( $P < 0.001$ ;  $n = 4$ ; Fig. 5-4D, F). Collectively, these data suggest that S-glutathionylation-mediated Kir6.1/SUR2B channel inhibition can be reversed substantially by deglutathionylation reagents.

### **5.3.6 Biochemical evidence for the Kir6.1 S-glutathionylation.**

Does S-glutathionylation occur on the channel protein or on another protein that regulates the channel activity? To address this question, we examined S-glutathionylation using membrane-permeable BioGEE. HEK293 cells transfected with Kir6.1/SUR2B or the expression vector alone were loaded with BioGEE (250  $\mu$ M) for 1 hr followed by an  $H_2O_2$  (750  $\mu$ M) challenge for 15 min. Clear labeling was observed in the Kir6.1/SUR2B-transfected cells (Fig. 5-6A-C), whereas a rather weak stain was seen with the mock transfection (data not shown). When the cells were double-stained with BioGEE (green) and Kir6.1 antibodies (red), co-localization was observed (Fig. 5-6D-F).

The Kir6.1 S-glutathionylation was further tested with the streptavidin pull-down assay (Zmijewski et al., 2009). The A10 vascular smooth muscle cell line, in which the Kir6.1/SUR2B channel was endogenously expressed (Shi et al., 2010; Sun et al., 2004), was loaded with BioGEE (250  $\mu$ M) for 1 hr followed by an  $H_2O_2$  (750  $\mu$ M) challenge for 15 min. A strong Kir6.1-reactive band ( $\sim 32$  kDa) was detected in the whole-cell lysate (Fig. 5-6G; lower panel). After pull-down with streptavidin, the cell lysate pretreated with a combination of BioGEE and  $H_2O_2$  showed a clear band of Kir6.1 immunoreactivity (Fig. 5-6G; upper panel), while no band

was observed in cell lysates treated with either BioGEE or H<sub>2</sub>O<sub>2</sub> alone (Fig. 5-6G). Similar results were obtained using HEK293 cells transfected with Kir6.1/SUR2B.

### **5.3.7 Inhibition of the K<sub>ATP</sub> channel activation by natural activators.**

Kir6.1/SUR2B channel activity is low under basal condition but increases significantly in the presence of several vasodilating hormones and neurotransmitters that are coupled to the adenylyl cyclase-cAMP-PKA pathway (Shi et al., 2008a; Shi et al., 2007b). Therefore, we examined the effect of S-glutathionylation on the Kir6.1/SUR2B currents activated by VIP (100 nM) and  $\beta$ -adrenoceptor agonist isoproterenol (Isop; 100 nM), both of which activate Kir6.1/SUR2B currents through the PKA pathway. In the whole-cell configuration, DTNP (50  $\mu$ M) or 2-DTP (50  $\mu$ M) inhibited Isop-activated currents strongly ( $94.1 \pm 3.6\%$  and  $97.0 \pm 0.9\%$ , respectively;  $n = 4$ ; Fig. 5-7A, C). Similarly, 2-DTP inhibited the VIP-activated currents by  $89.7 \pm 1.4\%$  ( $n = 3$ ; Fig. 5-7C). 2-DTP and DTNP were not acting at the level of the receptors as forskolin, an adenylyl cyclase activator, elicited currents that were also potently inhibited by these agents (Fig. 5-7C). In inside-out patches, basal currents as well as the currents activated by the catalytic subunit of PKA (cPKA; 50 units/mL), were strongly inhibited by 5 mM GSSG as well ( $79.1 \pm 5.4\%$ ,  $n = 4$  and  $89.1 \pm 5.0\%$ ,  $n = 4$ , respectively; Fig. 5-7B, C). Taken together, these results indicate that the Kir6.1/SUR2B channels are inhibited by S-glutathionylation, regardless of how the channels are activated.

## 5.4 Discussion

Inflammatory oxidative stress is a common pathogenesis of cardiovascular diseases, including hypertension, atherosclerosis, and diabetic vascular complications, which mostly result from the overproduction of ROS overwhelming the capacity of cellular antioxidant defense systems (Brownlee, 2001; Madamanchi et al., 2005). When excessively produced, ROS can cause damages to lipids, proteins, and nucleotides, leading to cell dysfunction, structural injuries, and death (Madamanchi et al., 2005). As the universal antioxidant treatment did not yield promising results in clinic trials (Kris-Etherton et al., 2004), the identification of specific molecules and the understanding of the molecular mechanisms underlying the oxidant-mediated protein modulation became crucial for the development of novel therapeutic strategies. Our studies indicate that the Kir6.1/SUR2B channel is inhibited by micromolar concentrations of  $\text{H}_2\text{O}_2$  through S-glutathionylation. Such channel inhibition is of pathophysiological relevance and is likely to occur in vasculatures as the production of micromolar concentrations of  $\text{H}_2\text{O}_2$  has been shown during oxidative stress (Ardanaz and Pagano, 2006).

$\text{H}_2\text{O}_2$ ,  $\text{O}_2^{\cdot-}$ , and  $\cdot\text{OH}$  are the major oxidants produced endogenously in biological systems. Their concentrations in the cytoplasm are tightly controlled by several antioxidant systems (Pryor et al., 2006). Early studies have shown that ROS including  $\text{H}_2\text{O}_2$  have modulatory effects on membrane proteins (Liu and Gutterman, 2002b; Tang et al., 2004; Zha et al., 2009). Most of the studies, however, use millimolar concentrations of  $\text{H}_2\text{O}_2$  (Ichinari et al., 1996; Krippeit-Drews et al., 1999). In the present study, we have observed a clear inhibition of the Kir6.1/SUR2B channel by  $\text{H}_2\text{O}_2$  with  $\text{IC}_{50}$  of  $\sim 1.5$  mM in whole-cell recording. In contrast, the inhibition of  $\text{K}_{\text{ATP}}$  currents can be clearly seen with micromolar concentrations of  $\text{H}_2\text{O}_2$  in vascular rings. The major reason for the discrepancy is likely to be the effect of washout or

inadequate controls of the cytosolic soluble factors that play a role in the channel modulation in oxidative stress. Indeed, some of the cytosolic substances are identified to be GSH and GSSG in the present study.

Searching for the missing cytosolic factors to better characterize the effect of  $\text{H}_2\text{O}_2$ , we conducted experiments in giant inside-out patches. We have found that supplying a small amount of exogenous GSH dramatically reduces the concentration of  $\text{H}_2\text{O}_2$  needed for channel inhibition. Using 2 mM GSH and 40  $\mu\text{M}$  GSSG, which mimic the intracellular GSH/GSSG ratio (Brown et al., 2007; Waypa et al., 2006), we have also observed clear channel inhibition by micromolar concentration of  $\text{H}_2\text{O}_2$ . The drastic effect of GSH/GSSG on the channel sensitivity to  $\text{H}_2\text{O}_2$  is not only seen in inside-out patches, but also takes place in the whole-cell recordings. A supplement of GSH/GSSG to the pipette solution markedly augments the  $\text{H}_2\text{O}_2$ -mediated inhibition of the whole-cell Kir6.1/SUR2B currents.

Comparing the inside-out patch data with the whole-cell recordings, we have found that the lower  $\text{H}_2\text{O}_2$  sensitivity in the whole-cell recordings appears to be also related to the transmembrane diffusion kinetics. The limited capacity of transmembrane diffusion may act as a cellular protection mechanism, diminishing or even eliminating the effect of a burst of  $\text{H}_2\text{O}_2$  production in the interstitial fluid. In the vascular inflammation state, however, such a membrane barrier does not seem adequate to protect the cell against a long-lasting production of  $\text{H}_2\text{O}_2$ . Consequently,  $\text{H}_2\text{O}_2$  manages to pass through the plasma membranes and inhibits the channel from inside with a potency similar to that seen in inside-out patches.

Such channel inhibition is not limited to  $\text{H}_2\text{O}_2$  as diamide, another oxidant, also produces strong channel inhibition when applied together with GSH. Furthermore, channel inhibition can be produced by the general S-glutathionylation-inducer GSSG. These results thus indicate that S-



glutathionylation is likely to be the underlying cause for Kir6.1/SUR2B channel inhibition by these oxidants under pathophysiological conditions.

Hence, several lines of evidence shown in the present study support S-glutathionylation of the Kir6.1/SUR2B channel. (i) The channel is inhibited by  $\text{H}_2\text{O}_2$  or diamide potently only when there is GSH. (ii) The S-glutathionylation inducer GSSG causes the channel inhibition. (iii) Several 2-PDSs react with thiol groups to form adducts at cysteine residues to inhibit the Kir6.1/SUR2B channel in micromolar concentrations. (iv) The oxidant-mediated channel inhibition can be reversed by the specific deglutathionylation reagent Grx1 and the general reducing reagent DTT. (v) Our pull-down assays reveal the incorporation of the GSH moiety to the Kir6.1 subunit in the presence but not in the absence of  $\text{H}_2\text{O}_2$ . This  $\text{K}_{\text{ATP}}$  channel modulation mechanism is not limited to the pinacidil-induced currents. The  $\text{K}_{\text{ATP}}$  currents activated by the natural vasodilators VIP and Isop are similarly inhibited by these oxidants.

The modulation of protein activity by S-glutathionylation is a newly recognized post-translational regulatory mechanism (Dalle-Donne et al., 2008; Dalle-Donne et al., 2009). This process, facilitated by oxidative stress and also seen in unstressed cells, can result in major changes to protein conformations and functions. Such modulation has been demonstrated in a large number of proteins using microarray (Fratelli et al., 2005) or proteomic analysis (Lind et al., 2002), while the physiological relevance of this modulation remains to be understood (Dalle-Donne et al., 2008). Several recent studies indicate that the following membrane proteins are modulated by S-glutathionylation: the cystic fibrosis transmembrane conductance regulator (Wang et al., 2005), the ryanodine receptor (Aracena-Parks et al., 2006), the sarco/endoplasmic reticulum  $\text{Ca}^{++}$  ATPase (Adachi et al., 2004), and the  $\text{Na}^+$ - $\text{K}^+$  pump (Figtree et al., 2009). The present study has shown for the first time that an important vascular tone regulator,

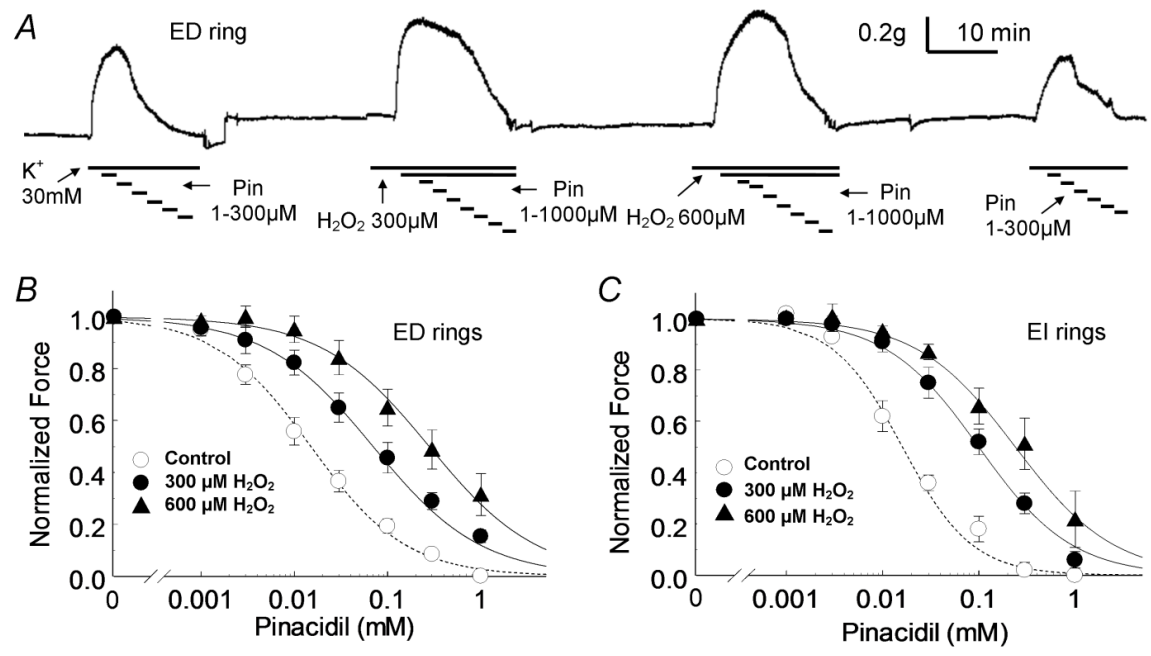
Kir6.1/SUR2B channel, is subject to S-glutathionylation that can be produced with  $H_2O_2$  at physiological or pathophysiological concentrations. This channel modulation by  $H_2O_2$  can lead to impairment of the vasodilation responses in a variety of vascular complications. Therefore, the demonstration of S-glutathionylation as a regulatory mechanism has important clinical implications. With the information, new therapeutic strategies may be formulated by preventing the oxidative modulation of the Kir6.1/SUR2B channel.

Another similar post-translational regulation mechanism is S-nitrosylation through which a nitric oxide (NO) moiety is incorporated into the thiol group of a cysteine residue (Martinez-Ruiz and Lamas, 2007; Yoshida et al., 2006). However, our initial attempts using NO donors yielded inconsistent results (data not shown).

In conclusion, our studies indicate that the Kir6.1/SUR2B channel is inhibited by  $H_2O_2$  at micromolar concentrations owing to S-glutathionylation in the Kir6.1 subunit. The demonstration of the mechanism underlying the impairment of the vascular  $K_{ATP}$  channel should have impacts on the treatment and prevention of several vascular diseases caused by oxidative stress.

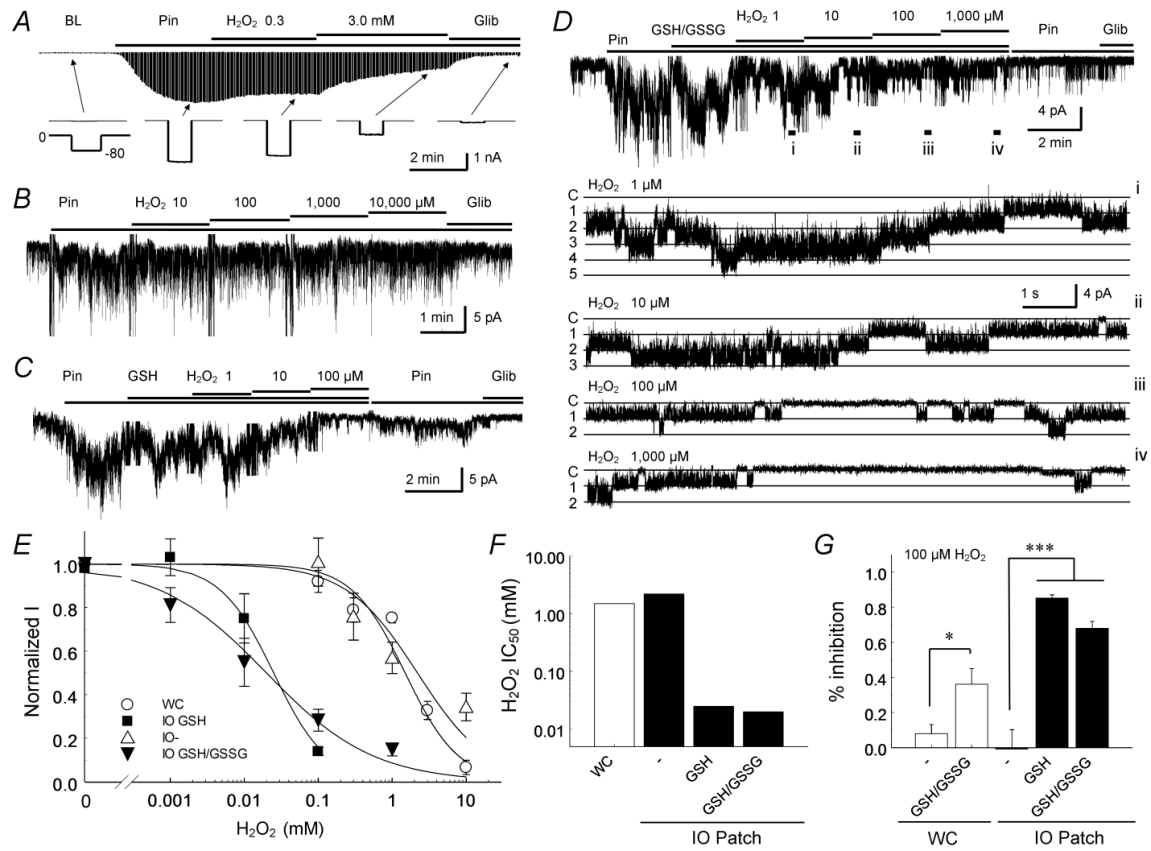
**Figure 5-1. The responses of mesenteric rings to  $K_{ATP}$  channel opener.** *A*, the relaxing effect of the  $K_{ATP}$  channel opener pinacidil (Pin) with and without  $H_2O_2$  pre-treatment were studied in a vascular endothelium-denuded (ED) ring obtained from mesenteric artery. The ring was contracted with 30 mM  $K^+$  followed by increasing concentrations of Pin. The constriction force was measured using a force-electricity transducer. Under basal conditions, Pin effectively relaxed the ring with a midpoint effect of  $\sim 10 \mu M$ .  $K^+$  and Pin were then washed out. After equilibration for 30 min, 300  $\mu M$   $H_2O_2$  was applied to the ring for 10 min, and the ring was constricted again using  $K^+$  followed by incremental concentrations of Pin. In this condition, the relaxing effect of Pin was impaired. About a 10-fold higher concentration of Pin was needed to relax the ring to the same 50% level. This experiment was then performed again with another dose of  $H_2O_2$  (600  $\mu M$ ) pretreatment followed by a final control round in which  $>80\%$  recovery in both constriction and relaxation was seen. *B*, in ED rings, the concentration-dependent vasodilation of Pin is shown in the presence and absence of  $H_2O_2$ . The data were described by the Hill equations with  $IC_{50}$  of 15  $\mu M$  in control ( $n = 11$ ), 70  $\mu M$  with 300  $\mu M$   $H_2O_2$  pretreatment ( $n = 4$ ), and 280  $\mu M$  with 600  $\mu M$   $H_2O_2$  pretreatment ( $n = 7$ ), respectively. *C*, similar experiments were also done in endothelium-intact (EI) rings with  $IC_{50}$  16  $\mu M$  in control ( $n = 6$ ), 100  $\mu M$  with 300  $\mu M$   $H_2O_2$  pretreatment ( $n = 6$ ), and 240  $\mu M$  with 600  $\mu M$   $H_2O_2$  pretreatment ( $n = 5$ ), respectively.

Figure 5-1



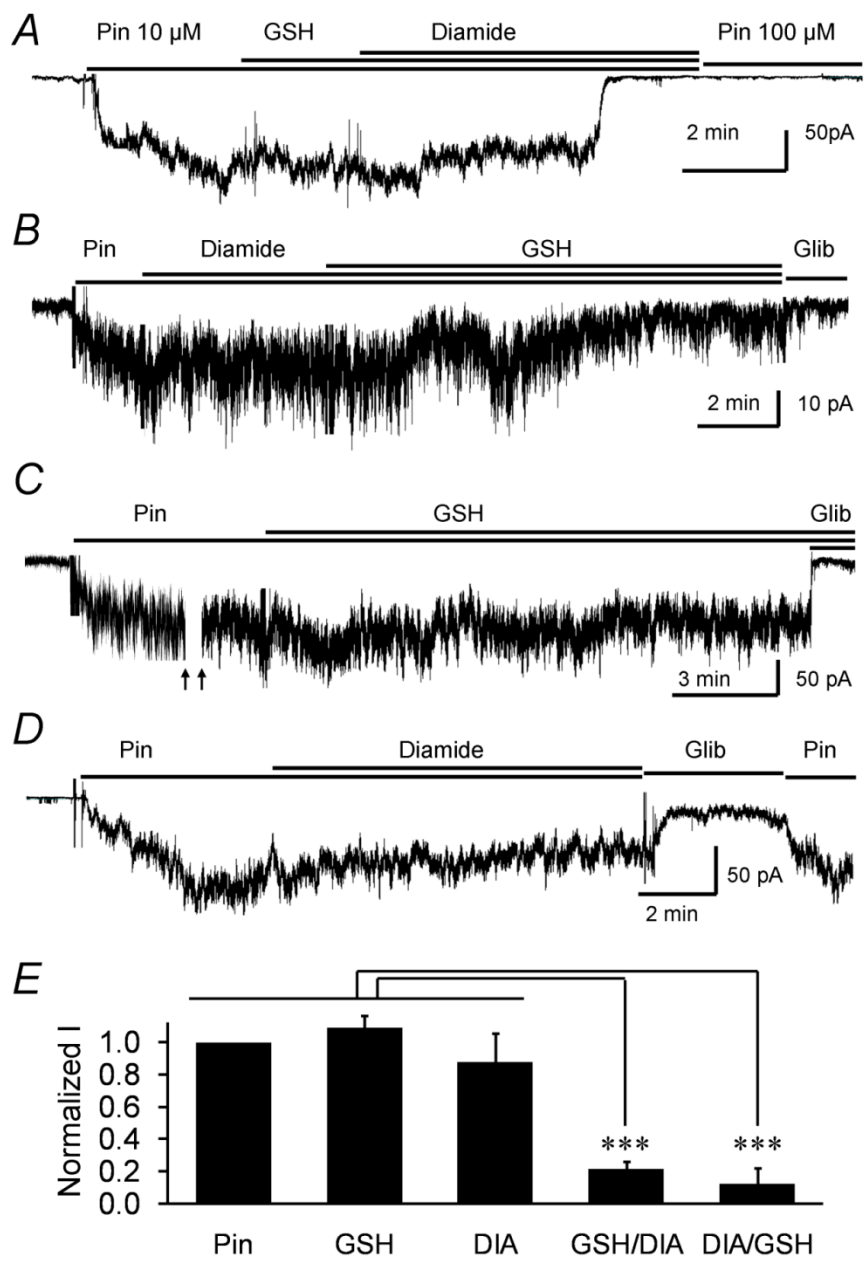
**Figure 5-2. Responses of vascular K<sub>ATP</sub> channel to H<sub>2</sub>O<sub>2</sub>.** *A*, whole-cell currents were recorded from a cell expressing Kir6.1/SUR2B channels. The bath solutions contained 145 mM K<sup>+</sup>, making the reversal potential of the K<sup>+</sup> currents close to 0 mV. Command pulses of –80 mV were given every 3 s. The recording pipette was filled with the same solution (bath solution) with the addition of 1 mM ATP, 0.5 mM ADP, and 1 mM free Mg<sup>++</sup>. Application of pinacidil (Pin, 10 μM) markedly augmented the whole-cell currents that were subsequently inhibited by 0.3 mM and 3 mM H<sub>2</sub>O<sub>2</sub> progressively. The K<sub>ATP</sub> channel specific blocker, glibenclamide (Glib; 10 μM) further reduced channel activity. The lower panel shows individual current traces produced by single command pulses. *B*, currents were recorded from giant inside-out patches obtained from HEK293 cells expressing the Kir6.1/SUR2B channel with a holding potential of –60 mV. Currents were activated by Pin, followed by increasing doses of H<sub>2</sub>O<sub>2</sub>. The H<sub>2</sub>O<sub>2</sub> exposures did not lead to significant changes in channel activity. *C*, in the presence of GSH (100 μM), the H<sub>2</sub>O<sub>2</sub> application strongly inhibited the Pin-activated currents, and the channels were almost completely inhibited by 100 μM H<sub>2</sub>O<sub>2</sub> in giant inside-out patches. *D*, similar channel inhibition by H<sub>2</sub>O<sub>2</sub> occurred in the presence of 2 mM GSH and 40 μM GSSG. Single channel activity is displayed in expanded records from the top trace. Five active channels were seen, which were dose-dependently inhibited by H<sub>2</sub>O<sub>2</sub>. Note that the channel retained the same conductance with H<sub>2</sub>O<sub>2</sub> exposure. Labels on the right: C, closure and 1, 2...5, the first, second ... fifth opening. *E*, dose-response relationships obtained in the whole-cell or inside-out patch configurations with or without GSH/GSSG. *F*, the half maximal inhibitory concentration (IC<sub>50</sub>) of H<sub>2</sub>O<sub>2</sub> was compared under whole-cell (WC) and inside-out patch (IO) configurations in the absence or presence of GSH or GSH/GSSG; *G*, the effect of 100 μM H<sub>2</sub>O<sub>2</sub> on the channel activity showed significant differences (\*, P < 0.05; \*\*\*, P < 0.001) between these conditions.

**Figure 5-2**



**Figure 5-3. The effect of diamide/GSH on the channel activity.** Currents were studied in giant inside-out patches obtained from HEK293 cells expressing the Kir6.1/SUR2B channel with a holding potential of  $-60$  mV. *A*, after channel activation by pinacidil, application of GSH ( $100\text{ }\mu\text{M}$ ) followed by diamide (DIA;  $100\text{ }\mu\text{M}$ ) for additional  $\sim 4$  min strongly inhibited the channel activity. The inhibition was not reversed by high concentration of pinacidil ( $100\text{ }\mu\text{M}$ ). *B*, in another cell, the application of DIA followed by GSH also markedly inhibited the Kir6.1/SUR2B channel activity. *C*, prolonged GSH ( $100\text{ }\mu\text{M}$ ) treatment did not have detectable effects on the Pin-activated currents. *D*, a prolonged treatment ( $8$  min) with DIA ( $100\text{ }\mu\text{M}$ ) did not cause marked channel inhibition. *E*, summary of the effects of Pin, GSH alone, DIA alone, GSH followed by DIA, and DIA followed by GSH.

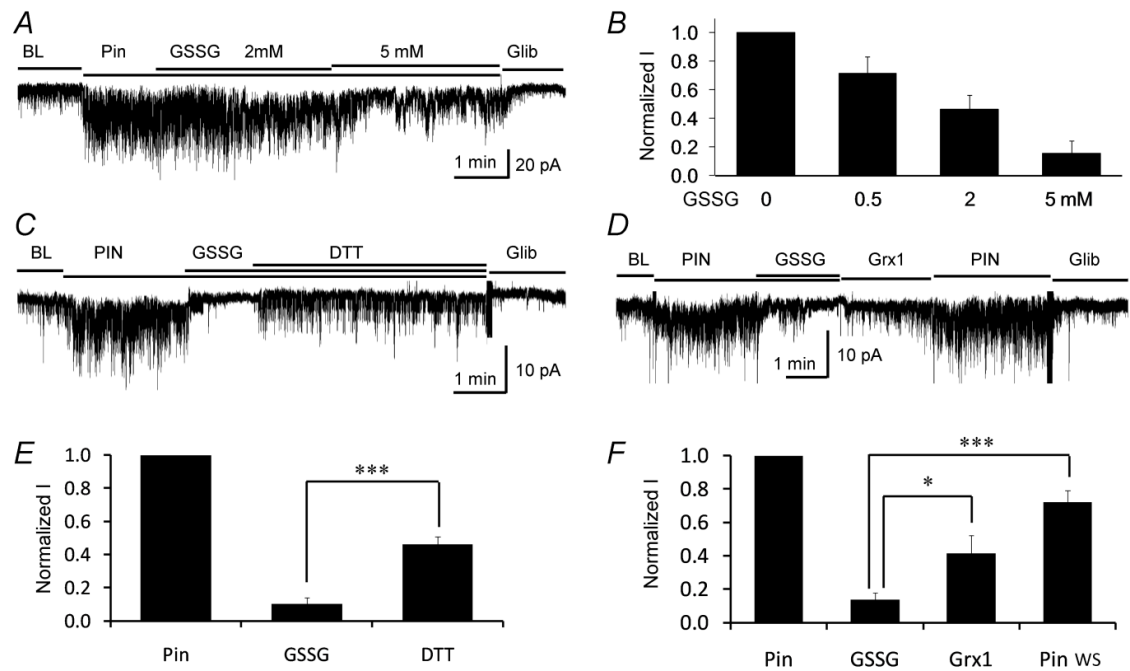
Figure 5-3





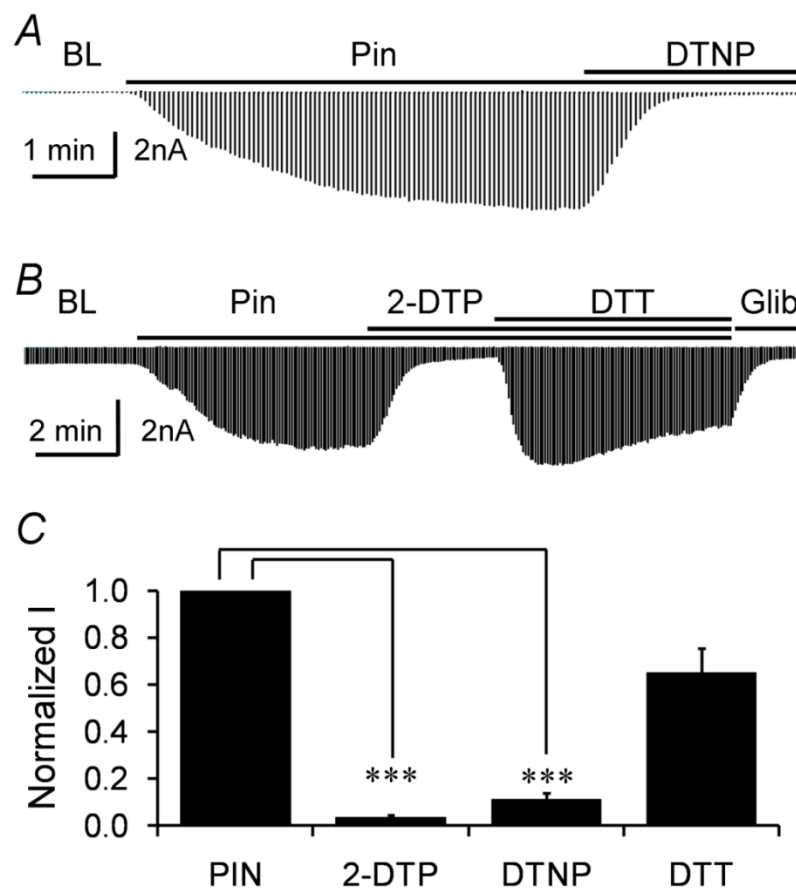
**Figure 5-4. The effect of GSSG on the channel activity and its reversibility.** *A*, in a cell expressing the Kir6.1/SUR2B channel, the Pin-activated currents were dose-dependently inhibited by GSSG treatment. *B*, summary of different dosages of GSSG on the channel activity ( $n = 4\sim 6$ ). *C*, in a giant inside-out patch, the Pin-induced currents were strongly inhibited by 5 mM GSSG. The current partially recovered with the application of the reducing agent (DTT, 5 mM). *D*, Pin-induced currents were inhibited by GSSG and partially reversed by 4  $\mu$ M Grx1. Additional pinacidil perfusion further augmented the channel activity. *E* and *F*, quantification of the data from *A* and *B*, respectively ( $n = 4$ ). \*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$ .

Figure 5-4



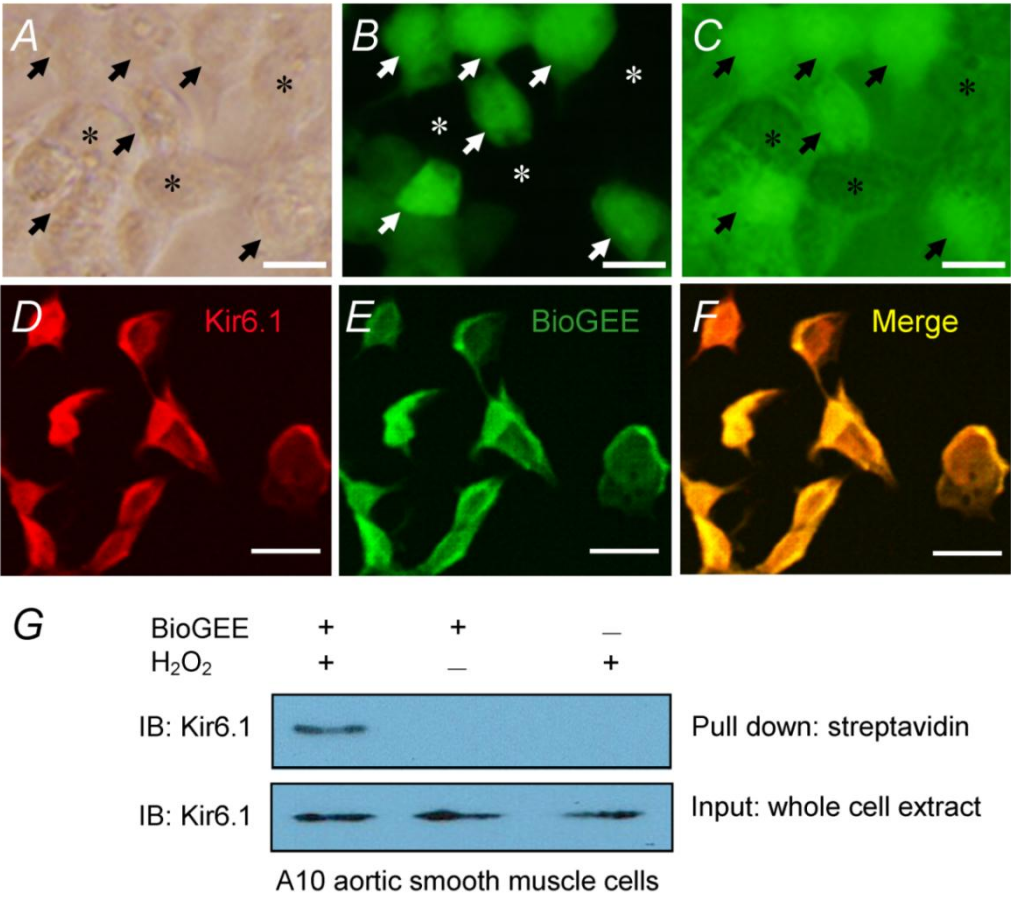
**Figure 5-5. Thiol modification by reactive disulfide reagents.** *A* and *C*, the chemical structures of 2-DTP and DTNP. *B*, in whole-cell configuration, the Pin-activated currents were potently inhibited by DTNP (50  $\mu$ M). *D*, another reactive disulfide, 2-DTP (50  $\mu$ M) also had a strong inhibitory effect on the Pin-sensitive currents of the Kir6.1/SUR2B channel, and this inhibitory effect was reversed by the reducing agent DTT (5 mM). *E*, the effects of 2-DTP (50  $\mu$ M), DTNP (50  $\mu$ M), and DTT (5 mM) on the channel activity are summarized. \*\*\*,  $P < 0.001$ .

**Figure 5-5**



**Figure 5-6. Biochemical detection of the Kir6.1-GSH interaction using BioGEE.** *A, B* and *C*, HEK293 cells transfected with Kir6.1/SUR2B were loaded with BioGEE for 1 hr and then challenged with H<sub>2</sub>O<sub>2</sub> (750  $\mu$ M) for 15 min. Free, unlabeled BioGEE was washed out, and the cells were visualized with streptavidin-Dylight 488 under phase contrast (*A*), florescence microscopy (*B*), and overlaid image (*C*). Labeled and unlabeled cells are indicated by arrows and asterisks, respectively. *D, E*, and *F*, 1.25- $\mu$ m confocal optical slices of HEK293 cells transfected with Kir6.1/SUR2B were double-stained with Kir6.1 (red) and BioGEE (green). Yellow indicates that Kir6.1 co-localizes with BioGEE. Scale bar = 25  $\mu$ m. *G*, the A10 aortic smooth muscle cells were lysed with RIPA buffer. Kir6.1 proteins were detected in the whole-cell lysis (lower panel). Only those protein samples that were obtained from the cells treated with both H<sub>2</sub>O<sub>2</sub> and BioGEE showed a clear band in the streptavidin pull-down assay (upper panel).

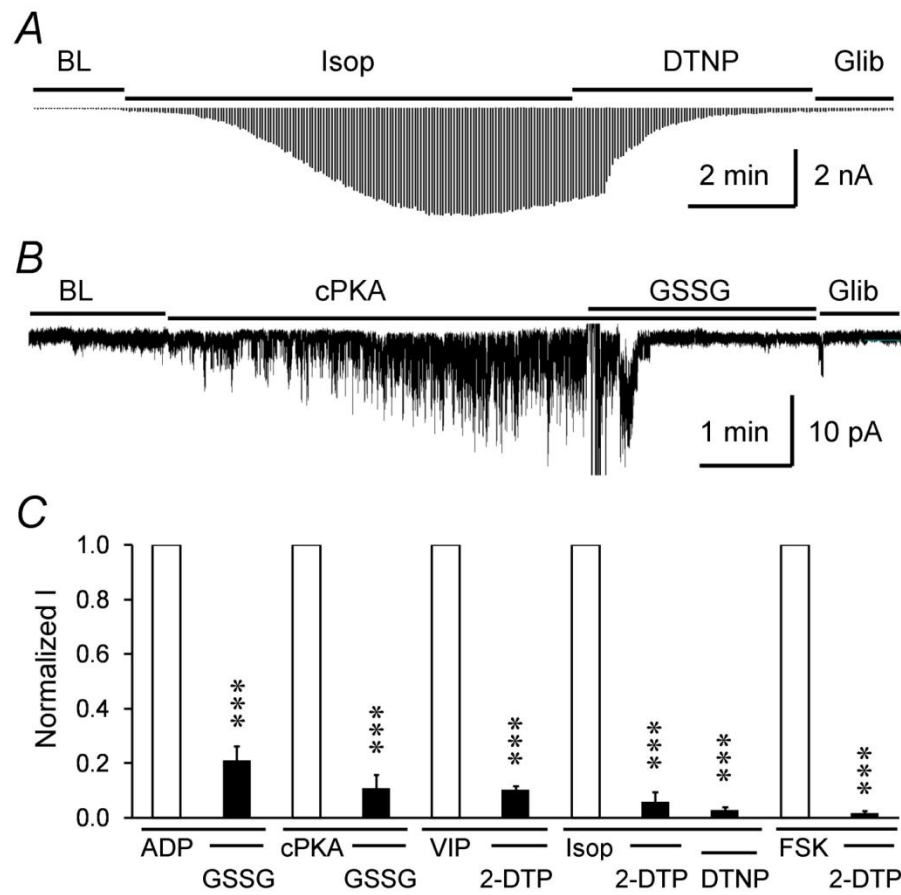
Figure 5-6



**Figure 5-7. Redox-mediated blockade of the  $K_{ATP}$  channel activation by natural activators.**

*A*, in whole-cell configuration, isoproterenol (Isop; 100 nM) activated the Kir6.1/SUR2B current that was subsequently inhibited by DTNP (50  $\mu$ M). *B*, in a giant inside-out patch, the channel was activated by the catalytic subunit of PKA (cPKA; 50 units/mL), and inhibited by 5 mM GSSG. *C*, summary of the effects of GSSG, DTNP, and 2-PDS on the Kir6.1/SUR2B channel induced by a variety of channel activators ( $n = 3\sim 5$ ). Abbreviations: FSK, forskolin; VIP, vasoactive intestinal polypeptide. \*\*\*,  $P < 0.001$ .

**Figure 5-7**





## 6. RESULT 2: MOLECULAR BASIS AND STRUCTURAL INSIGHT OF VASCULAR K<sub>ATP</sub> CHANNEL GATING BY S-GLUTATHIONYLATION

Publication: **Yang Yang\***, Weiwei Shi\*, Xianfeng Chen\*, Ningren Cui, Anuhya S. Konduru, Yun Shi, Timothy C. Trower, Shuang Zhang and Chun Jiang, Molecular basis and structural insight of vascular K<sub>ATP</sub> channel gating by S-glutathionylation, 2011, *J Biol Chem.* 286: 9298-9307

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Contribution disclosure: Yang Yang performed patch clamp experiments, biochemical assays (western blot and pull down experiments), cell culture, mutagenesis, protein structural modeling, data analysis, and wrote the paper draft. Weiwei Shi, Ningren Cui and Shuang Zhang assisted with patch clamp study. Xianfeng Chen contributes to the protein structural modeling. Anuhya S. Konduru contributes to the mutagenesis. Yun Shi did the chimeras construction. Timothy C. Trower did the cartoon illustration. Dr. Chun Jiang and Yang Yang designed and developed the research.

## 6.1 Abstract:

K<sub>ATP</sub> channel is targeted by a variety of vasoactive substances, playing an important role in vascular tone regulation. Our recent studies indicate that vascular K<sub>ATP</sub> channel is inhibited in oxidative stress via S-glutathionylation. Here we show evidence for the molecular basis of the S-glutathionylation and its structural impact on the channel gating. By comparing the oxidant responses of Kir6.1/SUR2B channel with Kir6.2/SUR2B channel, we found that Kir6.1 was the subunit responsible for oxidant sensitivity. Oxidant screening of Kir6.1-Kir6.2 chimeras demonstrated that the N-terminus and transmembrane domains of Kir6.1 were crucial. Systematic mutational analysis revealed three cysteine residues in these domains: Cys43, Cys120 and Cys176. Among them, Cys176 was prominent, contributing to >80% of the oxidant sensitivity. Kir6.1-C176A/SUR2B mutant channel, however, remained sensitive to both channel opener and inhibitor, which indicated that Cys176 is not a general gating site in Kir6.1 in contrast to its counterpart (Cys166) in Kir6.2. Protein pull-down assay with BioGEE showed that mutation of Cys176 impaired oxidant-induced incorporation of GSH into the Kir6.1 subunit. In contrast to Cys176, Cys43 had only modest contribution to S-glutathionylation, and Cys120 was modulated by extracellular oxidants but not intracellular GSSG. Simulation modeling of Kir6.1 S-glutathionylation suggested that after incorporation to residue 176, the GSH moiety occupied a space between the slide helix and two transmembrane helices. This prevented the inner transmembrane helix from undergoing conformational changes necessary for channel gating, retaining the channel in its closed state.

## 6.2 Introduction:

K<sub>ATP</sub> channels are expressed in a variety of tissues including smooth muscles, pancreatic  $\beta$ -cells, myocardium and neurons where they play an important role in cellular function (Nichols, 2006; Yokoshiki et al., 1998). Activity of the K<sub>ATP</sub> channels is tuned by physiological or pathophysiological stimuli including hypoxia, hyperglycemia, ischemia, and oxidative stress, allowing a regulation of cellular excitability according to the metabolic state (Miki and Seino, 2005). The vascular smooth muscle (VSM) isoform of K<sub>ATP</sub> channels regulates vascular tones (Seino, 1999; Teramoto, 2006). Activation of the channel by vasodilators produces hyperpolarization of VSM cells, reduces activity of the voltage-dependent Ca<sup>++</sup> channels, and relaxes VSMs. Inhibition of the channel leads to constriction of VSMs. Disruption of the vascular K<sub>ATP</sub> channel in mice results in vasospasm in coronary arteries and sudden cardiac death (Chutkow et al., 2002; Miki et al., 2002).

Other studies have further shown that disruption of the vascular K<sub>ATP</sub> channel has drastic effects on the systemic response to septic stress. With a forward genetic approach by genome-wide random chemical mutagenesis, Croker et al. (Croker et al., 2007) screened a large population of mice and found four strains that are highly susceptible to multiple septic pathogens including lipopolysaccharides (LPS). The LPS hypersensitivity phenotype of these mice is due to a null allele of Kcnj8, encoding the Kir6.1 subunit of vascular K<sub>ATP</sub> channel (Croker et al., 2007). Similar septic susceptibility has been observed in Kcnj8-knockout mice that also show coronary hypoperfusion and myocardial ischemia during LPS exposure. These studies thus indicate that the vascular K<sub>ATP</sub> channel not only contributes to the vascular tone regulation at physiological condition, but also affects critically systemic stress responses.

Our recent studies have shown that the vascular  $K_{ATP}$  channel is strongly inhibited in oxidative stress by S-glutathionylation (Yang et al., 2010). The S-glutathionylation is a post-translational modification mechanism occurring in a variety of physiological or pathophysiological conditions. This protein modulation mechanism is remarkable especially in vasculatures, as oxidative stress is a major contributing factor to several cardiovascular diseases, in which S-glutathionylation plays an important role (Dalle-Donne et al., 2007). Although S-glutathionylation is often associated with the adverse effects of oxidative stress, such a protein modulation is reversible under certain circumstances and can act as a functional modulation mechanism like protein phosphorylation. Thus, demonstration of how the S-glutathionylation affects protein function should have broad significance. As for the vascular  $K_{ATP}$  channel S-glutathionylation, the molecular basis remains unclear. Therefore, we performed the studies to elucidate the molecular basis and provide structural insight of the  $K_{ATP}$  channel S-glutathionylation.

## 6.3 Results

### 6.3.1 Subunit identification

Functional  $K_{ATP}$  channels consist of four pore-forming Kir6.x subunits (Kir6.1 or Kir6.2) and four regulatory sulfonylurea receptors (SUR1, SUR2A or SUR2B) (Seino, 1999; Yokoshiki et al., 1998). The Kir6.1/SUR2B channel is considered to be the major vascular isoform of the  $K_{ATP}$  channels, although Kir6.2 is also found in VSM cells (Cao et al., 2002; Tricarico et al., 2006). Our recent studies demonstrate that Kir6.1/SUR2B isoform of vascular  $K_{ATP}$  channel is targeted by ROS through S-glutathionylation (Yang et al., 2010). To further identify the specific subunit(s) or isoform(s) targeted by oxidants, we expressed Kir6.1/SUR2B and Kir6.2/SUR2B channels in HEK cells separately. Both Kir6.1/SUR2B and Kir6.2/SUR2B currents were activated by the specific  $K_{ATP}$  channel activator pinacidil (10  $\mu$ M), and inhibited by the channel inhibitor glibenclamide (Glib, 10  $\mu$ M; Fig. 6-1A, B). The currents of interest were normalized to the window of pinacidil and Glib effects.

Application of  $H_2O_2$  caused a marked inhibition of the Kir6.1/SUR2B currents in a concentration-dependent manner (Fig.6-1A, G). In contrast, the Kir6.2/SUR2B currents were barely inhibited by  $H_2O_2$  up to 10 mM ( $4.5 \pm 3.3\%$  inhibition,  $n=4$ ; Fig.6-1B, G). The differential responses to oxidants between Kir6.1/SUR2B and Kir6.2/SUR2B channels was further investigated using pyridine disulfides (PDSs), pharmacological tools that react with protein cysteine residues similarly as S-glutathionylation (Yang et al., 2010). 2,2'-Dithiodipyridine (2-DTP, 50  $\mu$ M), a membrane permeable PDS, inhibited whole-cell Kir6.1/SUR2B currents by  $95.8 \pm 1.1\%$  ( $n=5$ ; Fig. 6-1C,H), while the same concentration of 2-DTP inhibited the Kir6.2/SUR2B channel by only  $12.9 \pm 0.5\%$  ( $n=4$ ; Fig. 6-2B). This suggests that the critical residue(s) or domain(s) for thiol oxidation are located in Kir6.1.

### 6.3.2 Intra- versus extracellular location

To determine the intra- vs. extracellular location of the S-glutathionylation sites, the effects of membrane permeable and impermeable PDSs were compared. Under the same recording condition as for 2-DTP, a high concentration of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB, 200  $\mu$ M), a membrane impermeable PDS, marginally inhibited the channel activity by  $28.7 \pm 5.2$  % (n=5; Fig. 6-1D, H). These different effects of DTNB and 2-DTP were not due to different potencies of these PDSs, as 2-DTP and DTNB inhibited Kir6.1/SUR2B channel activity to almost the same extent when they were applied to the internal membranes of inside-out patches (Fig. 6-1E, F, I). The different effects of 2-DTP and DTNB in whole-cell recording versus inside-out patches therefore indicate that the modulation of thiol groups takes place mainly on the intracellular side of the plasma membranes.

### 6.3.3 Determination of critical protein domains

To determine the critical domains for channel inhibition, we took the advantage of the different sensitivity of the Kir6.1 and Kir6.2 to thiol oxidation, and performed experiments using six Kir6.1/Kir6.2 chimerical constructs created in our laboratory (Shi et al., 2008b). In these chimeras, the N-terminus, transmembrane core region and C-terminus were swapped between Kir6.1 and Kir6.2. All constructs expressed functional channels with SUR2B. The responses of these chimerical channels to 2-DTP were then examined. In comparison with 222 (or Kir6.2,  $12.9 \pm 0.5$ % inhibition, n=4; Fig. 6-2A, B), chimera 122 (the N-terminus of Kir6.2 was substituted with that of Kir6.1, and so are named for other chimeras) and 212 gained substantial sensitivity to 2-DTP with the N-terminus or core domain from Kir6.1 ( $68.5 \pm 6.2$ % and  $57.6 \pm 5.9$ % inhibition, respectively, n=4,  $p < 0.05$ ; Fig. 6-2A, C, D). However, chimera 221 did not show any significant difference in its response to 2-DTP compared with 222 (Fig. 6-2A, E,  $P > 0.05$ ).

Moreover, in comparison with 111 (or Kir6.1,  $95.8 \pm 1.1\%$  inhibition,  $n=5$ ), the inhibitory effect of 2-DTP on both 211 and 121 was significantly reduced ( $87.4 \pm 5.3\%$  and  $65.4 \pm 8.2\%$  inhibition, respectively,  $n=4$ ,  $p<0.05$ ; Fig. 6-2A). When both the N-terminus and the core domain exist, the 112 had 2-DTP sensitivity ( $91.9 \pm 8.0\%$  inhibition,  $n=4$ ) as great as the 111 (Fig. 6-2A). This chimerical analysis thus suggests that both N-terminus and core domain are crucial for the channel inhibition by 2-DTP.

### **6.3.4 Cys176 in the Kir6.1 subunit**

Systematic mutagenesis was carried out on all cysteine residues of the Kir6.1 (Fig. 6-3A, B). All the mutants exhibited functional channel activity when expressed with SUR2B in HEK cells. By 2-DTP screening, we found that one cysteine residue in the core domain (Cys176) and another in the N-terminus (Cys43) were important, mutations of which disrupted the channel sensitivity to 2-DTP ( $50 \mu\text{M}$ ) in whole-cell recordings (Fig. 6-3B-D). Mutation of any other cysteine residues did not result in significant change in 2-DTP sensitivity using ANOVA analysis (Fig. 6-3B). Of these two cysteine residues, the Cys176 was more prominent in the oxidant-mediated channel inhibition, as the Kir6.1-C176A/SUR2B channel lost most of its 2-DTP sensitivity ( $26.0 \pm 6.5\%$  inhibition,  $P<0.001$ ; Fig. 6-2B, C). Consistently, this mutant also lost its sensitivity to  $\text{H}_2\text{O}_2$  ( $3.5 \pm 4.7\%$  inhibition,  $P<0.001$ ).

The Kir6.1-C176A mutant was further tested with GSSG, an S-glutathionylation inducer that modulates Kir6.1/SUR2B channel in inside-out patches (Kil et al., 2008; Wang et al., 2005; Yang et al., 2010). The WT Kir6.1/SUR2B channel was inhibited by GSSG drastically ( $84.3 \pm 8.7\%$  inhibition,  $n=5$ ; Fig. 6-4E), while the Kir6.1-C176A/SUR2B channel was rather resistant to the GSSG treatments ( $17.8 \pm 6.9\%$  inhibition,  $n=4$ ,  $P<0.001$ ; Fig. 6-4A, E). A treatment with  $100 \mu\text{M}$   $\text{H}_2\text{O}_2$  in the presence of  $100 \mu\text{M}$  GSH, another S-glutathionylation

induction method (Yang et al., 2010), inhibited the Kir6.1-C176A mutant only marginally ( $14.7 \pm 6.4\%$  inhibition,  $n=4$ ,  $P<0.001$  compared with Kir6.1 WT; Fig. 6-4B, F) in inside-out patches. All the results were consistent with the idea that Cys176 was a critical site for S-glutathionylation.

### 6.3.5 Cys43 and Cys120 in oxidant sensitivity

The Cys43 mutation (C43A) reduced the channel sensitivity to 50  $\mu$ M 2-DTP ( $69.0 \pm 3.8\%$  inhibition,  $n=5$ ; Fig. 6-3B, D), although the effect was significantly smaller than that of Cys176 ( $P<0.001$ ). We further tested the effect of GSSG on C43A mutation and found that the response of the Kir6.1-C43A mutant to GSSG was comparable to its response to 2-DTP, showing rather modest inhibition of the channel activity ( $60.9 \pm 3.2\%$  inhibition,  $n=4$ ; Fig. 6-4C, E). When the Kir6.1-C176A/C43A double mutation channel was tested, we found that the GSSG effect was completely eliminated ( $-3.9 \pm 3.5\%$  inhibition,  $n=4$ ; Fig. 6-4D, E)

In addition to Cys176 and Cys43, Cys120 appeared to be important for the channel sensitivity to extra-cellular oxidants. The Kir6.1-C120S/SUR2B mutant channel lost substantial 2-DTP sensitivity ( $77.6 \pm 5.1\%$  inhibition,  $n=4$ ; Fig. 6-3E), although statistical significance was not found using ANOVA analysis. When the data were tested with student's t-test, however, its response to 2-DTP was significantly smaller than that of Kir6.1/SUR2B WT channel ( $P<0.01$ ). Therefore, further studies were conducted on this site. In inside-out patches, the Kir6.1-Cys120 mutant did not show any reduction in its sensitivity to GSSG in comparison to Kir6.1/SUR2B WT channel ( $87.0 \pm 5.8\%$  inhibition,  $n=4$  v.s.  $84.3 \pm 8.7\%$  inhibition,  $n=5$ , respectively,  $P>0.05$ ; Fig. 6-4E). When this mutant was examined in whole-cell recordings using membrane impermeable DTNB (200  $\mu$ M) applied extracellularly, the effect of DTNB was eliminated almost completely ( $2.7 \pm 6.0\%$  inhibition,  $n=6$ ; Fig. 6-3F) in comparison to the Kir6.1/SUR2B



WT channel ( $28.7 \pm 5.2\%$  inhibition by DTNB,  $n=5$ ; Fig. 6-1D). These data thus suggest that Cys120 may serve for extracellular redox sensing but does not seem to be an S-glutathionylation site.

### **6.3.6 Biochemical evidence for Cys176 modification**

As Cys176 was the dominating residue mediating the S-glutathionylation, we performed additional experiments to validate the essential role of Cys176 in S-glutathionylation biochemically. HEK cells transfected with WT channels or Kir6.1-C176A/SUR2B mutant were first tested for the expression of Kir6.1 protein. After cell lysis, anti-Kir6.1 antibodies detected strong Kir6.1 reactive bands in whole cell extracts in both constructs. According to our previous study, a strong reactive band of  $\sim 32$  kDa is Kir6.1-specific (Yang et al., 2010). The specificity of the antibodies is also confirmed by the internal experiments performed by Sigma-Aldrich. With  $\beta$ -actin as loading control, we found that the density of Kir6.1-C176A-reactive band was comparable with Kir6.1 WT band ( $106 \pm 8.5\%$ ,  $n=5$ ), indicating that the C176A mutation did not change the protein expression pattern of Kir6.1 subunit (Fig. 6-5A). These constructs thus were subjected to streptavidin pull-down assay. The HEK cells transfected with Kir6.1/SUR2B or Kir6.1-C176A/SUR2B were incubated with BioGEE (250  $\mu$ M) for 1 hr followed by 15 min  $H_2O_2$  (750  $\mu$ M) challenge as described previously (Yang et al., 2010; Zmijewski et al., 2009). If BioGEE was incorporated into the channel proteins, streptavidin-beads then should pull down the channel-BioGEE complex which would be further detected by Kir6.1 antibodies in western blot. On the other hand, if the mutation impaired the protein S-glutathionylation, then the mutation should decrease the binding of Kir6.1 protein to BioGEE, resulting in a weaker or even no band in western blot. Indeed, we observed different band densities between these two constructs: After streptavidin pull-down, the density of the Kir6.1-C176A-reactive band was

much weaker ( $38.6 \pm 3.8\%$ ,  $n=5$ ; Fig. 6-5A, B) compared with the band of Kir6.1 WT channel after normalizing to protein inputs (Fig. 6-5A, B). The data further demonstrate that the C176A mutation impaired the Kir6.1 protein S-glutathionylation.

As a control, the washout of the streptavidin bead-protein mixture was also used for western blot. No clear bands were detected in the washout from samples of either WT or mutant channels. This indicates that the Kir6.1 protein concentrations in the washout were below the threshold of detection by western blot.

### 6.3.7 Simulation modeling

Several Kir channel protein crystal structures including eukaryotic Kir2.2 channel structure were resolved recently. While N and C-termini show notable variation among different Kir channels, the core domains are rather conservative. To gain a structural insight into how the S-glutathionylation of Cys176 affects the channel activity, we modeled the Kir6.1 structure with a GSH moiety bound at residue 176 in its closed state (Fig. 6-6A, C-G). Our model showed that after incorporation, the GSH moiety occupied the space between the M0, M1 and M2 helices (Fig. 6-6C-G). Because the channel opening requires a movement of the M2 inner helix (Kuo et al., 2005; Kuo et al., 2003), the addition of GSH at residue Cys176 prevented the M2 inner helix from undergoing such a necessary movement for channel opening. To further validate this idea, we also modeled Kir6.1 structure in its open state (Fig. 6-6B, H) and compared it with Kir6.1 model in its closed state. In the Kir6.1 open state model, the M2 inner helix was bent and twisted, making the Cys176 residue facing the M1 outer helix directly (Fig. 6-6H). After energy minimization, the distance between Cys176 and the closest residue (Leu73) on the M1 outer helix was  $2.8\text{\AA}$  (Fig. 6-6H). When there was a GSH moiety on Cys176, channel open state with

such a short distance between Cys176 and Leu73 could not be achieved. Consequently, the channel was retained in its closed state.

## 6.4 Discussion

S-glutathionylation is a post-translational modulation mechanism that involves in a variety of physiological or pathophysiological events. We have recently shown that the VSM  $K_{ATP}$  channel, a vascular tone regulator, is modulated by S-glutathionylation. Our data in the present study reveal that the Kir6.1/SUR2B channel modulation by S-glutathionylation is likely to take place primarily at the Cys176 residue of the Kir6.1 subunit. Because of its critical location, the incorporation of GSH moiety to the Cys176 prevents the pore-forming inner helices from undergoing necessary conformational change for opening and retains the channel in its closed state.

Excessive ROS produced during oxidative stress can result in structural modification of proteins affecting protein function (Dalle-Donne et al., 2007). Although some studies have shown that the  $K_{ATP}$  channels are targeted by redox regulation, data are rather inconsistent regarding the effect of oxidants or thiol oxidation on  $K_{ATP}$  channels from different tissues. ROS lowers the  $K_{ATP}$  channel activity in cerebral arterioles (Erdos et al., 2004; Ross and Armstead, 2003) and coronary arteries (Miura et al., 2003) but facilitates its opening in cardiac myocytes (Tokube et al., 1998) and pancreatic  $\beta$ -cells (Krippeit-Drews et al., 1999). Our results suggest that the differential responses of  $K_{ATP}$  channels to ROS in these tissues are likely to be due to different isoforms of  $K_{ATP}$  channels expressed.

$K_{ATP}$  channels consist of Kir6.1-containing channels and Kir6.2-containing channels. Although the Kir6.2 is the closest family member of Kir6.1, the intrinsic properties of these two groups of channels are quite different (Hibino et al., 2010). For example, they have different

biophysical properties and are modulated differently by PKA and PKC (Light et al., 2000; Lin et al., 2000; Thorneloe et al., 2002); the sensitivities of these channels to ATP and ADP are distinct (Farzaneh and Tinker, 2008); without ATP, the Kir6.2 channel often opens automatically whereas the Kir6.1 channel always has low basal activity (Farzaneh and Tinker, 2008; Shi et al., 2008b; Trapp et al., 1998b). Therefore, the studies of Kir6.2 channel often take the advantage of its automatic opening whereas the studies of Kir6.1 channel require the  $K_{ATP}$  channel openers to activate the channel. In this current study, we found another major difference between these channels. The Kir6.1/SUR2B channel is inhibited by oxidants and reactive disulfides strongly whereas Kir6.2/SUR2B channel is barely inhibited by these reagents. These different responses may attribute to the difference between Kir6.1 and Kir6.2 subunits. However, we cannot rule out the possibility of the involvement of SUR subunit. The close coupling between Kir6.x and SURx is well recognized. The SUR is known to interact with the N-terminus but not the C-terminus of Kir6.x to affect channel gating (Babenko and Bryan, 2002, 2003). Such an interaction may rely on certain N-terminal residues and produce different conformational supports for distinct Kir6.x subunits. However, our data do suggest that the cysteine residues in the SUR2B subunit are not functionally S-glutathionylated, which is supported by our systematic mutagenesis for all the intracellular cysteine residues in SUR2B subunit (Fig. 6-7).

Previous studies of the oxidant sensitivity of  $K_{ATP}$  channels were mainly focused on Kir6.2-containing channels (Yokoshiki et al., 1998). Studies of Kir6.2-containing channels in native tissues suggest that the application of  $H_2O_2$  facilitates the opening of these channels, preferentially through changing the ATP/ADP sensitivity of the Kir6.2-containing channels (Ichinari et al., 1996; Krippeit-Drews et al., 1999). On the other hand, Kir6.2 $\Delta$ C26 (truncated Kir6.2 that can express by itself) or Kir6.2 with SUR1 is inhibited by p-

chloromercuriphenylsulphonate (pCMPS), a sulfhydryl-modifying reagent. Further investigations have shown that Cys42 (the corresponding site of Cys43 in Kir6.1) is the major site involved in the channel inhibition (Trapp et al., 1998b). Consistently, we have also found Cys43 in Kir6.1, the counterpart of Cys42 in Kir6.2, to be modulated by exogenous thiol oxidants. More importantly, this residue in Kir6.1 appears to be a S-glutathionylation site, although it does not seem to play a leading role compared with Cys176.

Biochemical experiments including western blot and pull-down assay require the use of Kir6.1 antibodies. Kir6.1 antibodies have been generated from different labs, and are also commercially available. Interestingly, although specific, antibodies from different sources detect bands of distinct sizes in different tissue samples. For example, a single band of 35, 44 or 49 kDa is detected in rat heart tissue from three independent studies (Akao et al., 1997; Morrissey et al., 2005; Sun et al., 2004), respectively. In rat brain samples, a single band of 51 kDa is detected (Sun et al., 2004), while another study detects the Kir6.1 band between 37 and 50 kDa in adult mouse hippocampus (Soundarapandian et al., 2007a). In the present study, we used the Kir6.1 antibodies purchased from Sigma-Aldrich. The size of Kir6.1 reactive-band detected in our study is close to the band detected from heart tissue by Sun et al (Sun et al., 2004). It is suggested that the post-translational modification, the natural truncation of Kir6.1 protein during the preparation and the methods for sample treatments (e.g. lysis reagents, reducing or non-reducing preparation, etc) may affect the size of Kir6.1 protein from different tissues.

K<sub>ATP</sub> channels have been modeled previously using crystallized bacterial channels as templates (Antcliff et al., 2005; Bichet et al., 2003; Bryan et al., 2004; Proks and Ashcroft, 2009). In this study the closed state of the Kir6.1 is modeled using the most recently crystallized eukaryotic Kir2.2 together with bacterial Kir channels as templates. The open state model is

generated based on the open state KirBac1.1 structural model provided by Dr. Venien-Bryan (Domene et al., 2005; Kuo et al., 2005). With the information from these structures, a model of the S-glutathionylation-mediated Kir6.1/SUR2B channel gating is proposed (Fig.6-6I-K). A conservative residue phenylalanine (Phe146 of KirBac1.1; Phe178 of Kir6.1) located in the narrowest region of the ion conduction pathway is likely to serve as “blocking residue/activation gate” that prevents  $K^+$  from passing through when the channel is closed (Kuo et al., 2003; Rojas et al., 2007). When the channel is open, the slide helix moves laterally, exerts strain on the bottom of the inner helix, resulting in the bending of the inner helix at a weak point, e.g. glycine residue (Gly175 of Kir6.1). This bending moves the side chain of the blocking residue (Phe178 of Kir6.1) away from the center of the ion-conduction pathway and allows  $K^+$  to pass through (Kuo et al., 2005). In the channel open state, the Cys176 of M2 helix makes a close contact with the M1 helix. The closest distance between Cys176 and Leu73 in the M1 helix is measured to be 2.8Å. Such a short distance/small space cannot accommodate a GSH moiety (or even a smaller thiol modulation reagent, 2-DTP). Therefore, when the channel is S-glutathionylated, the open conformation of the channel cannot be achieved.

In the Kir6.2 channel, Cys166 (corresponding to Cys176 in Kir6.1) is suggested to be involved in the intrinsic channel gating (Trapp et al., 1998a). The channel with the C166S mutation lost most of its sensitivity to both  $K_{ATP}$  channel opener and inhibitor with a drastic augmentation of the channel open probability (Trapp et al., 1998a). However, Cys176 in Kir6.1/SUR2B channel does not seem to be a general gating site. We have found that Kir6.1/SUR2B channels with the C176A mutation are still sensitive to both  $K_{ATP}$  channel opener and inhibitor, indicating that the general channel gating machinery of Kir6.1/SUR2B channel is still intact with the Cys176 mutation.

In contrast to the conserved core domain, the N-terminus of Kir channel shows considerable variations (Fig. 6-8). The crystal structure of N-terminus of Kir channels cannot be modeled with a decent resolution. Therefore, we did not attempt to study how the S-glutathionylation of Cys43 affects the protein conformation.

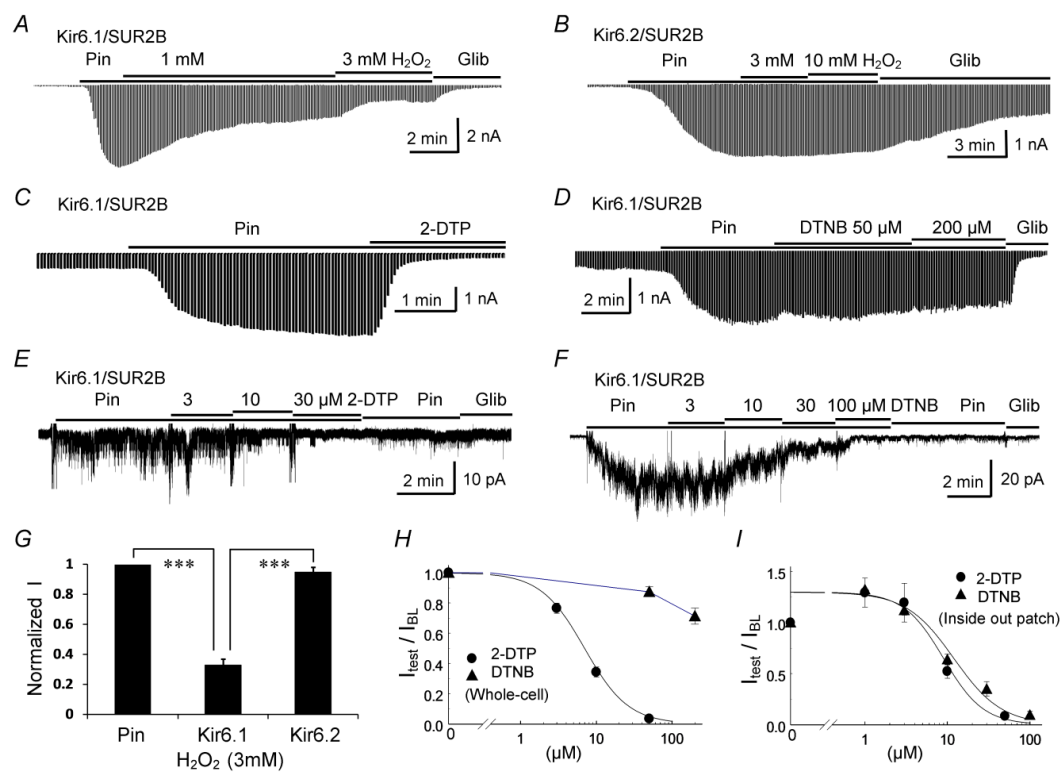
Unlike Cys176 and Cys43, Cys120 does not seem to be involved in S-glutathionylation. In the Kir2.1 channel, the mutation of Cys122, the counterpart of Cys120 in Kir6.1, results in an absence of ionic currents even the channels are still expressed (Cho et al., 2000; Leyland et al., 1999). In our studies, we were able to record the currents from the C120S mutant although the currents were rather small compared to most of the other mutants. Based on the Kir protein structure, Cys120 is located on the extracellular interface of the cellular membranes, accessible to extracellular environments. The accessibility of this residue to extracellular oxidants, as well as membrane-impermeable PDS but not intracellular GSSG, suggests that Cys120 could be a site for extracellular redox modulation rather than intracellular S-glutathionylation.

In conclusion, our studies indicate that S-glutathionylation inhibits the Kir6.1/SUR2B channel by targeting mainly Cys176 in the Kir6.1 subunit. S-glutathionylation at Cys176 is likely to structurally prevent the pore-forming inner helix from undergoing necessary conformational change for channel gating, thus retaining the channel in its closed state. The demonstration of the molecular mechanism underlying S-glutathionylation of the vascular  $K_{ATP}$  channel with structural insight into the channel gating should have a profound impact on the understanding of the post-translational modifications of ion channels.

**Figure 6-1. Redox-mediated vascular K<sub>ATP</sub> channel inhibition.** A, whole-cell voltage clamp was performed on a cell expressing Kir6.1/SUR2B channel. The reversal potential of K<sup>+</sup> current was close to 0 mV by using equilibrate bath and pipette solutions containing 145 mM K<sup>+</sup>. Command pulses of – 80 mV were given every 3s. Application of pinacidil (Pin, 10 μM) markedly augmented the whole-cell currents, which were subsequently inhibited by increased doses of H<sub>2</sub>O<sub>2</sub> (1 mM and 3 mM). Glibenclamide (Glib, 10 μM), a specific K<sub>ATP</sub> channel inhibitor, further diminished the channel activity to the basal level. B, in the whole cell recording, Pin-induced Kir6.2/SUR2B currents responded poorly to high concentrations of H<sub>2</sub>O<sub>2</sub> (3 mM and 10 mM). C, in whole cell configuration, the Pin-activated currents were potently inhibited by membrane-permeable 2-DTP (50 μM). D, in whole-cell configuration, membrane-impermeable DTNB (50, 200 μM) had modest inhibitory effects on the Kir6.1/SUR2B channel activity. E, inside-out patch recording was obtained from an HEK cell expressing the Kir6.1/SUR2B channel with a holding potential of – 60 mV, 2-DTP (3~30 μM) inhibited the Pin-induced Kir6.1/SUR2B channel progressively. F, increased doses of DTNB inhibited the Kir6.1/SUR2B channel in a similar manner. G, the effects of H<sub>2</sub>O<sub>2</sub> (3 mM) on the Kir6.1/SUR2B and Kir6.2/SUR2B channels were compared. H, the dose-effect relationship of extracellular application of 2-DTP or DTNB on the activity of Kir6.1/SUR2B channel in the whole-cell configuration was shown. I, the dose-effect relationship of 2-DTP or DTNB application on the activity of Kir6.1/SUR2B channel in inside-out patch configuration was shown. \*\*\*, P<0.001.

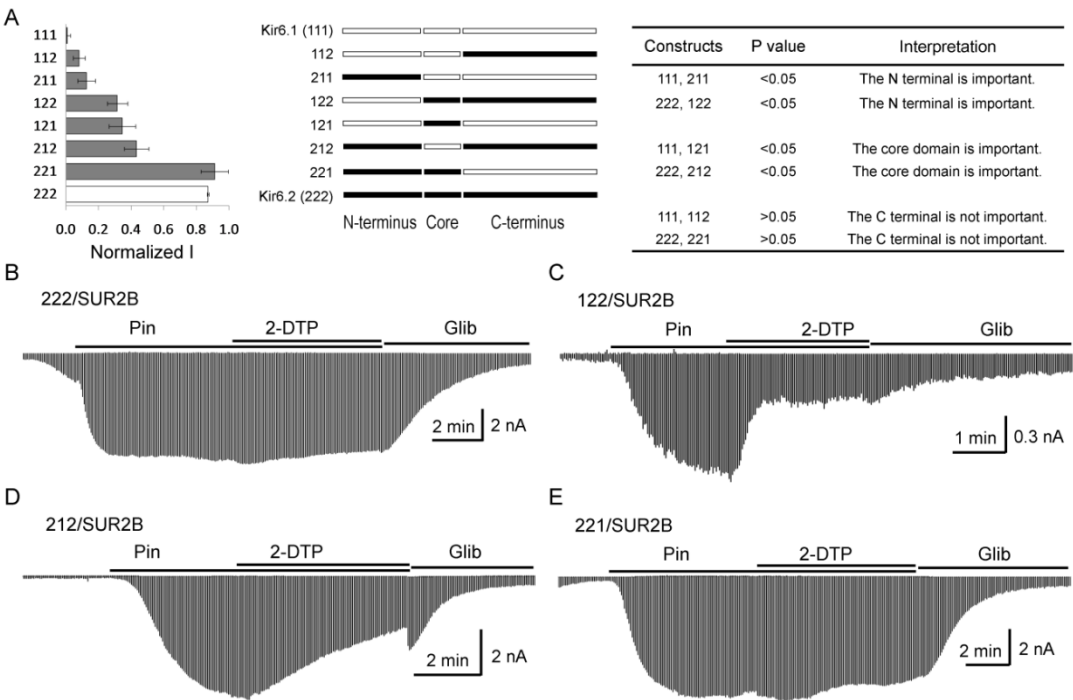


Figure 6-1



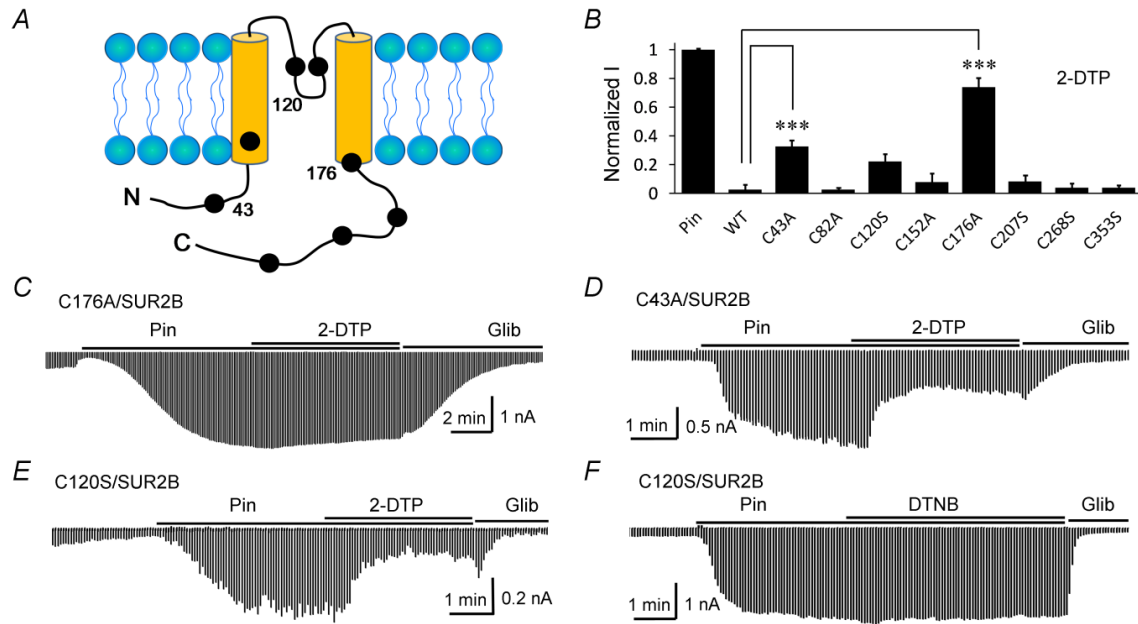
**Figure 6-2. The responses of chimerical channels to 2-DTP.** A, segments from Kir6.1 were shown in white and segments from Kir6.2 were shown in black. The effect of 2-DTP (50  $\mu$ M) on all the chimerical channels were shown along with the illustrations. Compared with 111, both 211 and 121 showed decreased sensitivities to 2-DTP. Compared with 222, both 122 and 212 had stronger responses to 2-DTP treatments. Statistical analysis revealed that both N-terminus and core domain were important for channel inhibition by 2-DTP. B-E, representative figures demonstrated the responses of different constructs to the treatment of 2-DTP.

Figure 6-2



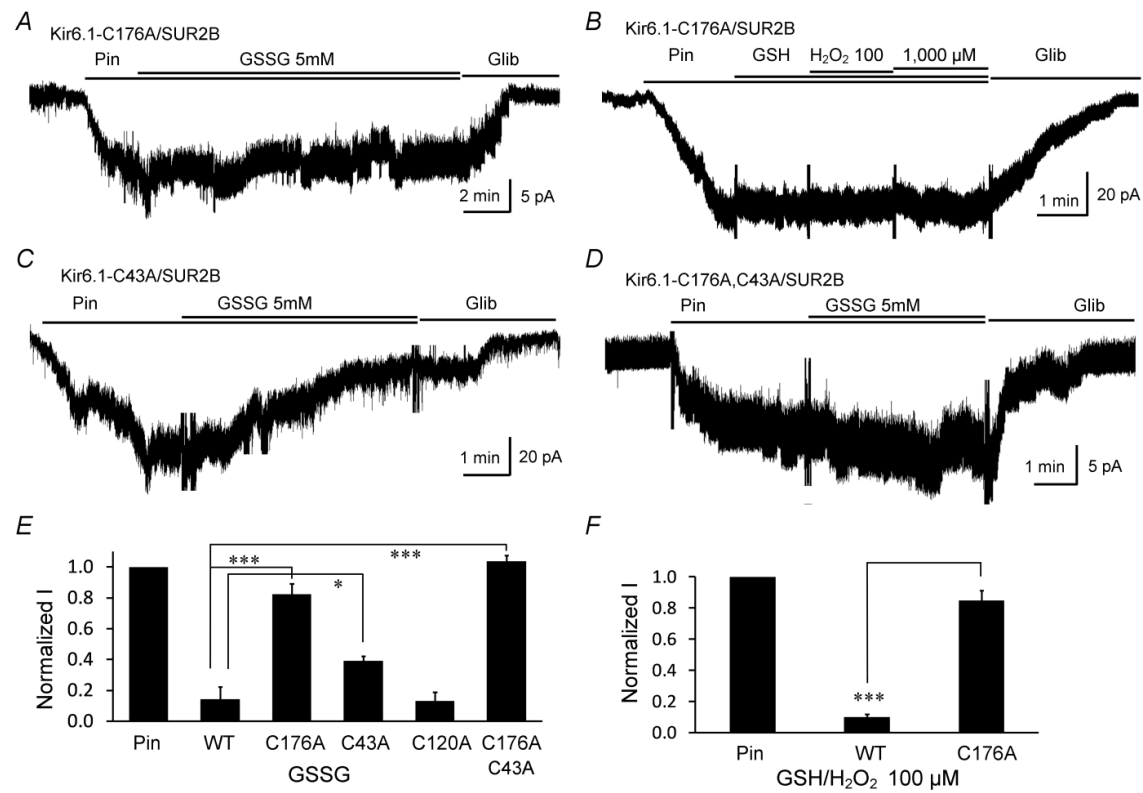
**Figure 6-3. Identification of the critical sites of cysteine modification.** A, schematic illustration of the approximate locations of the cysteine residues across the hKir6.1 subunit was shown. B, The effects of 2-DTP (50  $\mu$ M) on the WT and mutant channels were summarized. Mutation of Cys43 and Cys176 showed impaired sensitivities to the 2-DTP (50  $\mu$ M) treatment using ANOVA analysis. C, Kir6.1-C176A/SUR2B mutant channel was activated by Pin. The current was resistant to the inhibitory effect of 2-DTP. Glib still inhibited the channel activity. D, Kir6.1-C43A/SUR2B mutant channel showed impaired responses to 2-DTP-mediated channel inhibition. E, in another cell expressing Kir6.1-C120S/SUR2B mutant channel, 2-DTP had substantially inhibitory effect on the channel activity. F, Kir6.1-C120S/SUR2B currents were not sensitive to DTNB (200  $\mu$ M), a membrane impermeable 2-PDS (\*\*\*,  $P < 0.001$ ).

**Figure 6-3**



**Figure 6-4. The effect of S-glutathionylation on the WT and mutant channels.** A, in Kir6.1-C176A/SUR2B mutant channel, 5 mM GSSG only had a slight inhibitory effect on the Pin-induced currents in giant inside-out patches. B, Kir6.1-C176A/SUR2B mutant channel was almost insensitive to the combined treatment of GSH and H<sub>2</sub>O<sub>2</sub>, another S-glutathionylation induction method. C, Kir6.1-C43A/SUR2B mutant channel was inhibited by GSSG (5 mM). D, Kir6.1-C43A/C176A/SUR2B channel was resistant to the inhibitory effect of GSSG. E, summary of the effects of GSSG on Cys176, Cys43, Cys120 and Cys43/Cys176 double mutants in giant inside-out patches was shown. (n=4~6). F, summary of the effects of GSH/ H<sub>2</sub>O<sub>2</sub> on Kir6.1/SUR2B WT and Kir6.1-C176A/SUR2B mutant channels was shown. \*, P<0.05; \*\*\*, P<0.001.

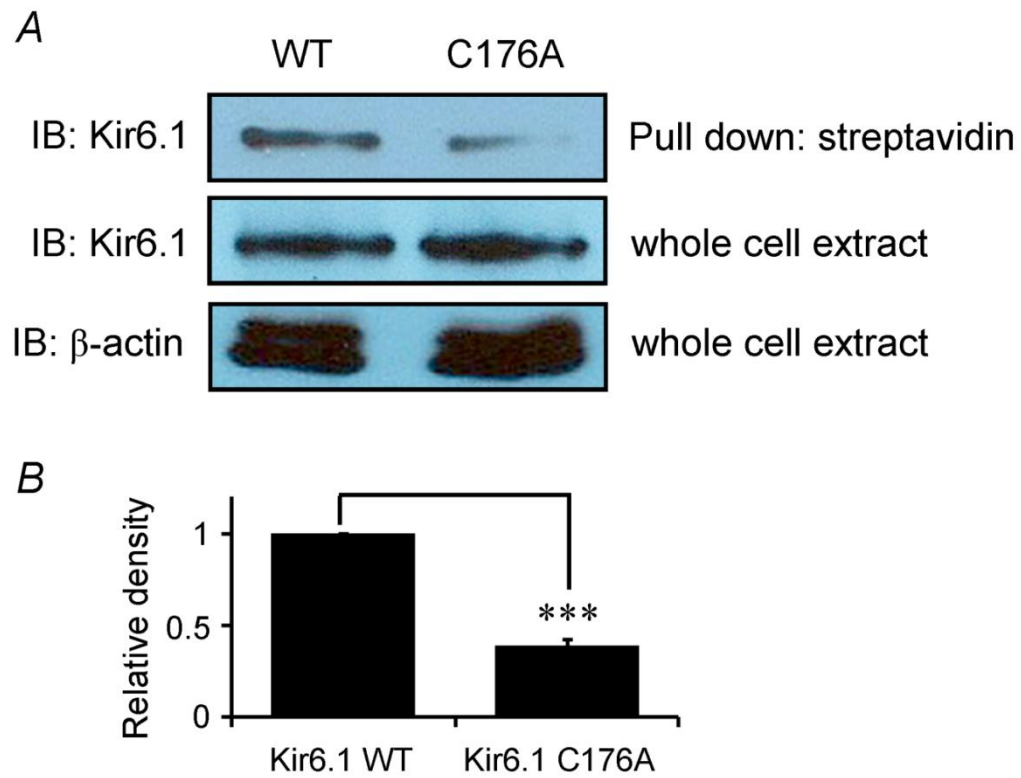
Figure 6-4



**Figure 6-5. Cys176 impaired the streptavidin pull-down.** A, whole cell extracts were obtained from HEK cells expressing Kir6.1/SUR2B and Kir6.1-C176A/SUR2B channels using RIPA buffer. The  $\beta$ -actin protein was detected using anti- $\beta$ -actin antibodies as the loading control (lower panel). The Kir6.1 proteins and Kir6.1-C176A mutant proteins were detected in the whole cell extracts using anti-Kir6.1 antibodies. With the loading of the same amount of protein extracts, the anti-Kir6.1 antibodies detected bands of Kir6.1/SUR2B WT channel and Kir6.1-C176A/SUR2B mutant channel in a similar density (middle panel). Same amount of protein extracts (100  $\mu$ g) from HEK293 cells expressing WT or mutant channels were subjected to the pull-down assay by same amount of streptavidin-beads (1 mg). Western blot using antibodies against Kir6.1 protein showed that the density of the Kir6.1-C176A-reactive band was much weaker compared with that of Kir6.1 WT channel (upper panel). B, The band densities of Kir6.1-C176A mutant channel after pull down was normalized to that of Kir6.1 WT channel with the adjustment of inputs. Data was averaged from five individual repeats. \*\*\*,  $P < 0.001$ .

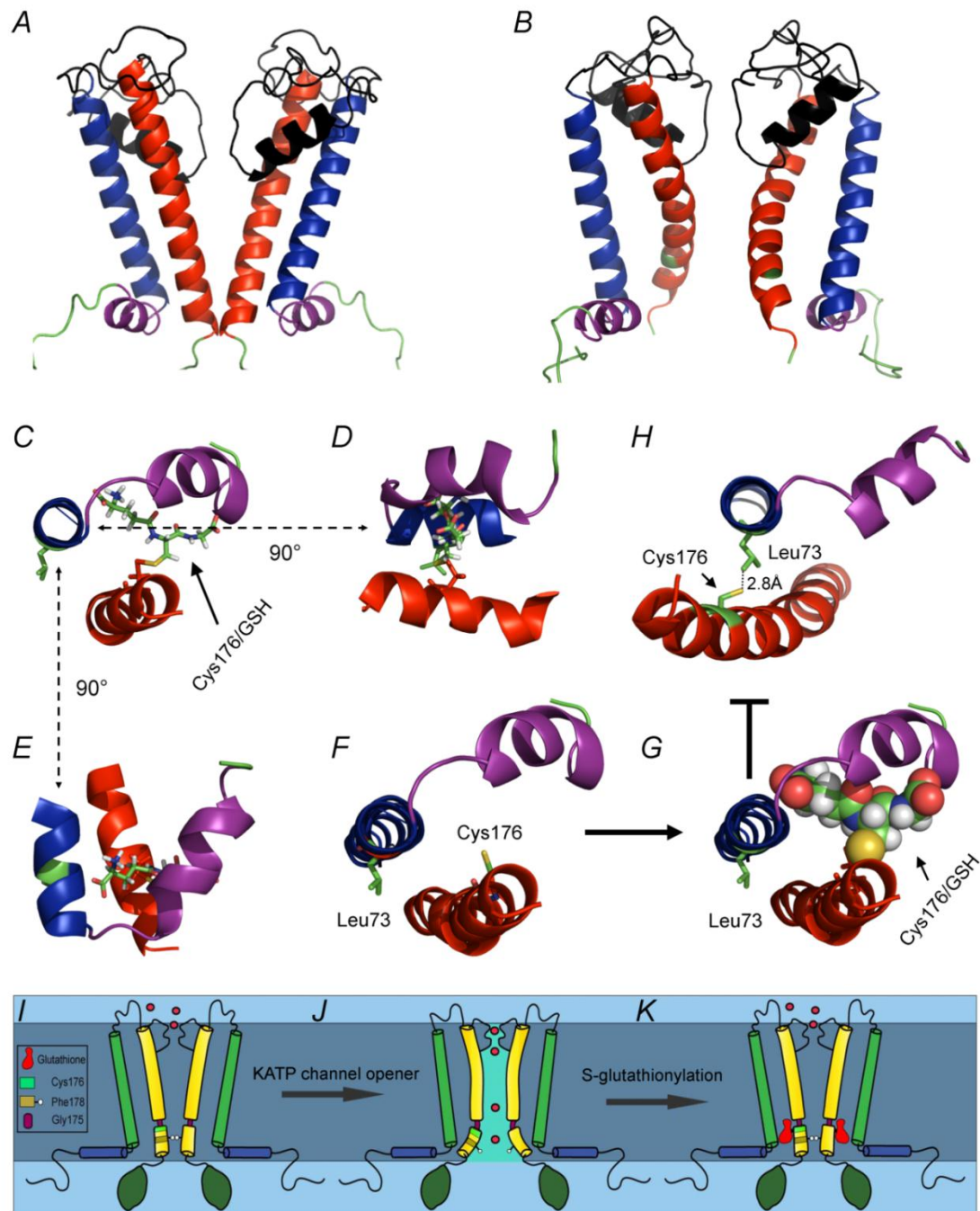


**Figure 6-5**



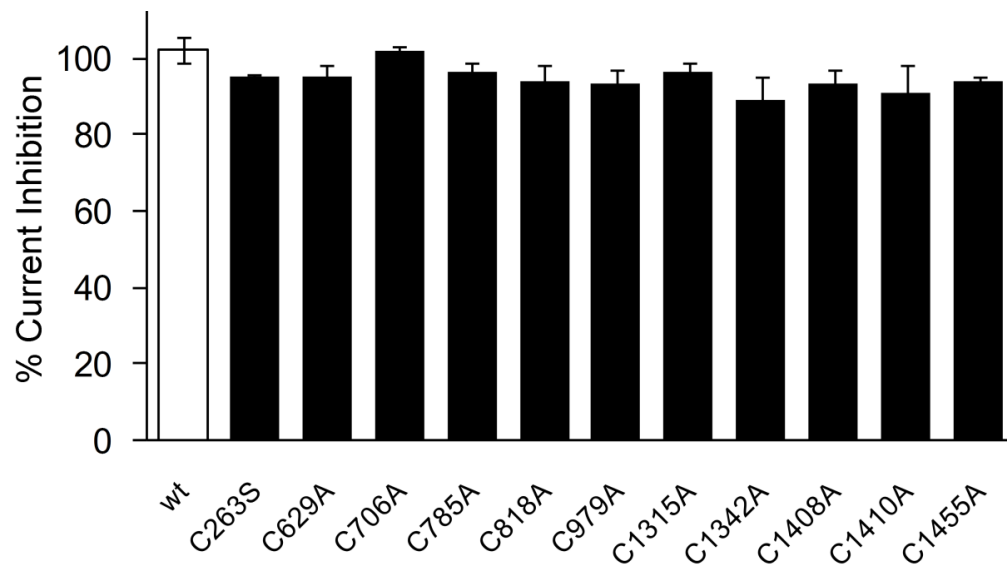
**Figure 6-6. Structural modeling of Kir6.1 S-glutathionylation at Cys176.** A, structural modeling of the two opposing monomers of Kir6.1 pore-forming domain in the closed state was shown. B, structural model of the Kir6.1 pore-forming domain in its open state was shown. C-E, close-up views of the GSH incorporated into Cys176 of M2 inner helix were shown. D and E were 90 degree turns of the view from C. F-G, intracellular view of the pore-lining helices of Kir6.1 (perpendicular to the axis of M1 outer helix, blue) in the closed state model. (G, with a GSH moiety; F, without a GSH moiety). H, the same intracellular view of Kir6.1 in the open state model was shown. Compared with F, the M2 inner helix (red) was bent leftward and twisted slightly, making Cys176 facing directly to the M1 outer helix (blue) where there was the residue Leu73. The distance between the sulfur atom of Cys176 and the closest carbon atom of Leu73 was 2.8Å in the open state compared with 9.2Å in the closed state. Structural elements are colored: blue, M1 outer helix; red, M2 inner helix; purple, M0 slide helix; black, selectivity filter; GSH, in standard amino acid colors. I-K, proposed models for S-glutathionylation mediated channel gating. I, schematic view of K<sub>ATP</sub> channel in its closed state. The “activation gate” Phe178 blocks the pore and prevents the ion from passing through. J, the administration of K<sub>ATP</sub> channel opener causes a conformational change of K<sub>ATP</sub> channel: Inner helix bends at Gly175 and moves the gate (Phe178) away from the ion-conduction pathway to facilitate potassium ions to pass through. K, S-glutathionylation at Cys176 residue results in the incorporation of GSH that occupies the space between inner and outer helices. The addition of GSH prevents the channel from entering its open state.

**Figure 6-6**



**Figure 6-7 Systematic mutagenesis of cysteine residue in SUR2B subunit of Kir6.1/SUR2B channel.** The mutation of all intracellular cysteine residues of SUR2B was created and expressed with wild-type Kir6.1. The mutants were tested with 2-DTP (50  $\mu$ M). None of these mutants acted differently from the wild-type Kir6.1/SUR2B channels.

**Figure 6-7**



**Figure 6-8 Sequence alignments of hKir6.1 with KirBac1.1, KirBac3.1 and cKir2.2.** M0 slide helix was colored gray, M1 outer helix was colored pink; selectivity filter was colored dark yellow; M2 inner helix was colored red. Important residues of Kir6.1 described in this study were also highlighted: Gly175, green; All cysteine residues, yellow; Phe178, teal.

**Figure 6-8**

```

KirBac1.1 -----MNVDPFSPHSSDSFAQAASPARKPPRGRR IWS-GTREVIAYGMP 44
Kirbac3.1 -----MTGGMKPPARKP-----RI LNSDGSSNI TRLGLE 29
hKir6.1 MLAR---KSI IPEEY---VLARIAAEN-LRKPRI RDR-LPKARF IAKSGA NLAHKNIR 51
cKir2.2 MTAGRVNPYS IVSSEEDGLRLTTMP GINGFGNGKI HTRRKC RNRFVKKN GQC NVEFTNMD 60
                                         :      * : . * :: .:

      Slide helix      Outer Helix
KirBac1.1 AS--VWR-DLYYWALKVSWPVFFASLAALFVVNNTL FALLYQLGDAPIANQ----- 92
Kirbac3.1 KR--GWLDDHYHDLLTVSWPVFITLITGLYLV TNALFALAYLACGDVIENA----- 78
hKir6.1 EQ--GRFLQDI FTTLVDLKWRHTLVI FTMSFLC SWLLFAIMWWLVFAFAHGD IYAYMEKSGM 110
cKir2.2 DKPQRYIADMFTTCVDIRWRYMLLLFSLAFLVSWLLFGLI F WLIALIHGDL---ENPGG 116
      : * : : : * : :: : . **:: : :

      Pore Helix      Filter      Inner Helix
KirBac1.1 -----SPPGFVGAFFFSVETLATVGYG--DMHPQTVYAHAIATLEIFVGM SGI 138
Kirbac3.1 -----RPGSFTDAFFFSVQT MATIGYG--KLIPISPLANTLVLTLEALCGMLGL 124
hKir6.1 EKSGLESTVGV TNVRSFTSAFLFSIEVQVTIGEGGRMMTEECPLAITVLILQNI VGLIIN 170
cKir2.2 DDT--FKPCVLQVNGFVAAFLFSIETQTTIGYGERCVTEECP LAVFMVVQSI VGCIIID 173
      . * . **::**::.. .*:** : : * : : : : *

KirBac1.1 ALSTGLVFARFARPR---AKIMFARHAIVRPFNGRMTLMVRAANARQNVIAEARAKMRLM 195
Kirbac3.1 AVAASLIYARFTRPT---AGVLFSSRMVISDFEGKPTLMMLRLANLRIEQIIEADVHLVLV 181
hKir6.1 AVMLGCIEMKTAQAHRAETLIFSRHAVIAVRNGKLCFMRVGD LRKSMIISASVRIQVV 230
cKir2.2 SFMIGAIMAKMARPKKRAQTLLFSHNAVAMRDGKLC LMW RVGNLRKSHIVEAHVRAQLI 233
      :. . : : :. :*: . : : :*: :* * .: * . * . * .: : :

KirBac1.1 RREHSSEG-YSLMKI HDLKLVRNE--HPIFLLG-WNMMHVIDESSPLFGETPESLAEGRA 251
Kirbac3.1 RSEISQEG-MVFRRFHDLTLTRSR--SPIFSLS-WTMHPIDHHSPIYGETDET LRNSHS 237
hKir6.1 KKT TTPEGEVVPIHQLDIPVDNPIESNNIFLVAPLIICHVIDKRSPLYDISATDLANQDL 290
cKir2.2 KPRITEEGEYIPLDQIDIDVGFDKGLDRIFLVSPITILHEINEDSPLFGISRQDLETDDF 293
      : : ** :*: : ** :. : * *:. **::. : *

KirBac1.1 MLLVMIEGSDETTAQVMQARHAWEHDDIRWHHRYVDLMSDV-DGMTHIDYTRFNDTEPVE 310
Kirbac3.1 EFLVLFTGHHEAFAQNVHARHAYSCDEIIWGGHFVDVFTTLPDGRRALDLGKFHEIAQH 297
hKir6.1 EVIVILEGVVETTGIT TQARTSYIAEEIQWGH RFVSIVTEE-EGVYSVDYSKFGNTVKV- 348
cKir2.2 EIVVILEGMVEATAMTTQARSSYLASEILWGH RFEPVLFEE-KNQYKVDYSHFHKTYEVP 352
      .:*** * *: . **: : .:* * : : . . . * :* .

KirBac1.1 PP GAA-----PDAQAF AAKPGEGDARPV----- 333
Kirbac3.1 HHHH----- 301
hKir6.1 AAPRC SARELDEKPS ILIQTLQKSEL SHQNSLRKRNSMRRNNSMRRNNS IRRNNS SLMVP 408
cKir2.2 STPRCSAKDLVEN-KFLLPSTNSFCYENELAFMSRDEDEEDDDSRGLDD LSPDNRHEFDR 411

KirBac1.1 -----
Kirbac3.1 -----
hKir6.1 KVQFMTPEGNQNTSES-- 424
cKir2.2 LQATIALDQRSYRRESEI 429

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**7. RESULT 3: PKA-DEPENDENT ACTIVATION OF THE VASCULAR SMOOTH  
MUSCLE ISOFORM OF K<sub>ATP</sub> CHANNELS BY VASOACTIVE INTESTINAL  
POLYPEPTIDE AND ITS EFFECT ON RELAXATION OF THE MESENTERIC  
RESISTANCE ARTERY**

Publication: **Yang Y**, Shi Y, Guo S, Zhang S, Cui N, Shi W, Zhu D, Jiang C. PKA-dependent activation of the vascular smooth muscle isoform of K<sub>ATP</sub> channels by vasoactive intestinal polypeptide and its effect on relaxation of the mesenteric resistance artery, 2008, ***Biochim Biophys Acta-Biomembranes***. 1778: 88-96

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Contribution disclosure: Yang Yang performed patch clamp experiments, cell and tissue culture, data analysis, and wrote the paper draft. Shouli Guo and Shuang Zhang performed the vascular ring experiment. Dr. Daling Zhu supervised the vascular ring study. Yun Shi, Ningren Cui and Weiwei Shi assisted with patch clamp study. Dr. Chun Jiang and Yang Yang designed the research.



## 7.1 Abstract

Vasoactive intestinal polypeptide (VIP) is a potent vasodilator, and has been successfully used to alleviate hypertension. Consistently, disruption of VIP gene in mice leads to hypertension. However, its downstream targets in the vascular regulation are still not well demonstrated. To test the hypothesis that the vascular smooth muscle isoform of  $K_{ATP}$  channels is a downstream target of the VIP signaling, we performed the studies on the Kir6.1/SUR2B channel expressed in HEK293 cells. We found that the channel was strongly activated by VIP. Through endogenous VIP receptors, the channel activation was reversible and dependent on VIP concentrations with the midpoint-activation concentration  $\sim 10$  nM. The channel activation was voltage-independent and could be blocked by  $K_{ATP}$  channel blocker glibenclamide. In cell-attached patches, VIP augmented the channel open-state probability with modest suppression of the single channel conductance. The VIP-induced Kir6.1/SUR2B channel activation was blocked by PKA inhibitor RP-cAMP. Forskolin, an adenylyl cyclase activator, activated the channel similarly as VIP. The effect of VIP was further evident in the native tissues. In acutely dissociated mesenteric vascular smooth myocytes, VIP activated the  $K_{ATP}$  currents in a similar manner as in HEK293 cells. In endothelium-free mesenteric artery rings, VIP produced concentration-dependent vasorelaxation that was attenuated by glibenclamide. These results therefore indicate that the vascular isoform (Kir6.1/SUR2B) of  $K_{ATP}$  channels is a target of VIP. The channel activation relies on the PKA pathway and produces mesenteric arterial relaxation.

## 7.2 Introduction

VIP is a 28-amino-acid peptide hormone and neurotransmitter present in multiple organs and systems. VIP has broad effects on cellular functions including vasodilation, water reabsorption, neurotransmission, insulin secretion and immunomodulation (Delgado et al., 2001; Straub and Sharp, 1996; Tsutsumi et al., 2002). These biological effects are mediated by specific VIP receptors (VPAC1 and VPAC2), both of which are coupled to G-proteins, primarily the  $G_s$  proteins (Laburthe and Couvineau, 2002).

As a potent vasodilator, VIP containing nerve terminals innervate a variety of blood vessels in systemic and pulmonary circulations (Henning and Sawmiller, 2001). VIP released from the nerve terminals produces vascular smooth muscle relaxation. Such a vasodilation effect is 50-100 times more potent than acetylcholine (Henning and Sawmiller, 2001). Administration of VIP to patients with severe cardiovascular diseases such as primary pulmonary hypertension results in substantial improvement in their conditions without adverse side-effects (Petkov et al., 2003). Moderate pulmonary arterial hypertension has been observed in mice lacking the VIP gene (Hamidi et al., 2006; Said et al., 2007). By taking the advantage of the vasodilation effects, several species of animals have developed VIP-like peptides that are lethal vasodilatory toxins serving for defense and predatory purposes (Blank et al., 1986; Hoshino et al., 1984) (Pohl and Wank, 1998).

The downstream molecules of VIP in the vasodilation effect are still not fully understood (Henning and Sawmiller, 2001). In the vascular smooth muscle cells, the influx of  $Ca^{++}$  through voltage-dependent  $Ca^{++}$  channels contributes to vessel constriction, while the preclusion of this event leads to the vasodilation (Hill et al., 2001; Nilsson, 1998). The opening and closure of these  $Ca^{++}$  channels is largely controlled by membrane potentials. Activation of the vascular

K<sub>ATP</sub> channels hyperpolarizes the vascular smooth muscle cells, prevents the Ca<sup>++</sup> influx, and relaxes blood vessels. Therefore, the vascular K<sub>ATP</sub> channels play an important role in vascular tone regulation (Brayden, 2002).

Functional K<sub>ATP</sub> channels are made of four pore-forming subunits Kir6.x (Kir6.1 or Kir6.2) and four regulatory subunits sulfonylurea receptor SURx (SUR1, SUR2A or SUR2B) (Quayle et al., 1997). The former belongs to the inwardly rectifying K<sup>+</sup> channels, and the latter is a member of the ATP-binding cassette (ABC) protein family (Babenko et al., 1998; Deeley et al., 2006; Seino, 1999). A combination of different Kir6.x and SURx results in distinct K<sub>ATP</sub> channels, such as the Kir6.2/SUR1 channel in pancreatic  $\beta$ -cells and the Kir6.2/SUR2A channel in cardiac muscles (Yokoshiki et al., 1998). The Kir6.1/SUR2B channel is the major isoform of K<sub>ATP</sub> channels in vascular smooth muscles, although there is evidence that the Kir6.2 also forms functional channels with SUR2B in blood vessels (Cao et al., 2002; Jansen-Olesen et al., 2005; Tricarico et al., 2006). The biophysical and pharmacological properties of the Kir6.1/SUR2B channel are comparable to those of K<sub>NDP</sub> channels found in native coronary and mesenteric arteries (Zhang and Bolton, 1995; Zhang and Bolton, 1996). Consistently, disruptions of the *Kcnj8* (Kir6.1) or *ABCC9* (SUR2) genes in mice cause abnormalities in coronary circulation, sudden cardiac death and fatal susceptibility to endotoxemia (Chutkow et al., 2002; Kane et al., 2006; Miki et al., 2002).

Previous studies have suggested that the vascular K<sub>ATP</sub> channels are subjected to phosphorylation regulation by protein kinases A, C and G (PKA, PKC, PKG), allowing them to respond to several vasoactive hormones and neurotransmitters (Quinn et al., 2004; Shi et al., 2007a; Shi et al., 2007b; Thorneloe et al., 2002). Since the PKA signaling cascade can be activated by VIP, it is possible that the vascular smooth muscle K<sub>ATP</sub> channels play a role in

vasodilation effect of VIP. To test the hypothesis, we performed these studies on the Kir6.1/SUR2B channel expressed in HEK293 cells, cell-endogenous  $K_{ATP}$  channels from dissociated smooth myocytes and isolated mesenteric arterial rings.

## 7.3 Result

### 7.3.1. Activation of the Kir6.1/SUR2B channel by VIP in HEK293 cells

The Kir6.1/SUR2B channel was transiently expressed in the HEK293 cells, and whole cell voltage-clamp was performed on the GFP-positive cells. The cell was exposed to an extracellular perfusion solution after whole-cell configuration formation. Most cells showed small stable baseline currents, and the currents in some cells increased slightly reaching a steady state within a few minutes (Fig.7-1A). When VIP (30 nM) was applied to the cell in the perfusion solution, the whole-cell currents increased rapidly and reached a plateau in about 2-3 min.  $K_{ATP}$  channel opener pinacidil (Pin, 10  $\mu$ M) strongly augmented the currents that was subsequently inhibited by glibenclamide (Glib, 10  $\mu$ M) (Fig. 7-1A). Thus we used 10  $\mu$ M Pin and 10  $\mu$ M Glib throughout the study unless otherwise stated. Another  $K_{ATP}$  channel opener, diazoxide (100  $\mu$ M), opened the channel to the similar degree as Pin, and its effect was also totally blocked by Glib (Fig.7-8). The effect of VIP was completely blocked in the presence of 10  $\mu$ M Glib suggesting that Kir6.1/SUR2B channel is targeted by VIP (Fig. 7-1B). For quantitative analysis, the effect of VIP was normalized between the baseline current and the current activated by 10  $\mu$ M Pin. Under such a condition, the currents activated by 30 nM VIP averaged  $58.0 \pm 3.4\%$  (n=5).

Several control experiments were done. The outward currents studied together with the inward currents using depolarizing ( $-80$  mV) and hyperpolarizing (80 mV) command pulses were affected by VIP and other  $K^+$  channel blockers in a similar proportion as the inward currents (Fig. 7-8; Fig. 7-9). Both the Pin-activated inward and outward currents were insensitive to 30  $\mu$ M 4-aminopyridine (4-AP) and 100 nM charybdotoxin (ChTX), specific blockers of  $K_v$  and BK channels, respectively, but inhibited by 1 mM tetrabutylammonium (TBA) (Fig. 7-9).

Finally, the Pin- and Glib-sensitive currents were not observed in cells transfected with the expression vector alone (Fig.7-3B), indicating that the VIP-elicited current is conducted through  $K_{ATP}$  channels.

The activation of the Kir6.1/SUR2B channel by VIP showed clear concentration-dependence. Evident increase in the current amplitude was observed with the VIP concentration as low as 1 nM, and stronger activation occurred with higher concentrations (Fig. 7-2A). The maximum effect ( $58.0 \pm 3.4\%$ ) was reached with 30 nM VIP (Fig. 7-2B).

Previous studies have shown that the VIP receptor VPAC1 is constitutively expressed in HEK293 cells (Reubi, 2000; Simmons, 1990). We found that the Kir6.1/SUR2B channel was activated by VIP when expressed in HEK293 cells without exogenous VIP receptors. Such a channel activation was significantly diminished by the VIP receptor antagonist VIP6-28 ( $16.1 \pm 3.9\%$  10 nM VIP with the presence of VIP 6-28 vs  $35.0 \pm 7.9\%$  10 nM VIP without VIP 6-28;  $P < 0.05$ ,  $n = 15$ ) (Fig. 7-2C). When the HEK293 cells were transfected with the VPAC2 receptor (the VPAC1 receptor cDNA is not commercially available), VIP (100 nM) did not show any significant additional effect on the Kir6.1/SUR2B channel ( $49.2 \pm 4.5\%$  with the VPAC2 receptor vs.  $52.8 \pm 3.4\%$  without;  $P > 0.05$ ,  $n = 7$ ). These results suggest that the endogenous VIP receptors in the HEK293 cells mediate the VIP effect, consistent with previous observations on the presence of endogenous VIP receptors in HEK293 cells (Simmons, 1990).

### **7.3.2. Biophysical basis for the Kir6.1/SUR2B channel activation**

When step voltage protocols were applied, the current activation by VIP was observed across the whole voltage range from -120 mV to 120 mV (Fig.7-3A). The I-V relationship of the VIP-activated and Pin-activated currents was normalized to the current amplitude at -120 mV

and plotted in the same X-Y axis system. Under the condition, the two I-V plots superimposed with each other nicely, indicating that the current activation by VIP is voltage-independent (Fig. 7-3B, C). Such currents were not seen in cells transfected with the expression vector alone (Fig. 7-3D).

To understand the biophysical basis for the Kir6.1/SUR2B channel activation, single-channel recordings were performed in cell-attached patches. Exposure to VIP (100 nM) augmented the channel open-state probability ( $NP_o$   $0.005 \pm 0.002$  at baseline,  $0.048 \pm 0.006$  with VIP, and  $0.116 \pm 0.017$  with Pin,  $n=5$ ) (Fig. 7-4A). The single-channel conductance was slightly inhibited by VIP ( $34.9 \pm 0.9$  pS before vs.  $31.7 \pm 1.7$  pS after VIP treatment,  $P < 0.05$ ,  $n=10$ ; Fig. 7-4B,C). At baseline, the closed time was  $164.7 \pm 74.1$  ms ( $n=6$ ), the level-1 open time was  $1.77 \pm 0.53$  ms ( $n=6$ ), and the level 2 open time was  $0.73 \pm 0.19$  ms ( $n=6$ ). During the VIP exposure, the closed time was reduced to  $59.1 \pm 7.6$  ms ( $n=7$ ,  $P < 0.05$ ), while the level 1 open time ( $2.28 \pm 0.45$  ms,  $n=7$ ) and level 2 open time ( $1.06 \pm 0.15$  ms,  $n=7$ ) did not change significantly, indicating that VIP increases the  $NP_o$  via suppression of the closed time.

### 7.3.3. PKA dependence

Previous studies have shown that  $K_{ATP}$  channels are regulated by both PKA and PKC (Beguin et al., 1999; Lin et al., 2000; Quinn et al., 2004). VIP receptors (VPAC1 and VPAC2) are primarily coupled to  $G_s$ , stimulation of which can lead to activation of PKA pathway (Laburthe and Couvineau, 2002; Martin Shreeve, 2002). To elucidate whether the activation of the Kir6.1/SUR2B channel by VIP depends on the PKA pathway, 8-(4-chlorophenylthio) adenosine-3',5'-cyclic monophosphorothioate Rp-isomer (Rp-cAMP), a PKA inhibitor, was applied in both the pipette solution (200  $\mu$ M) and in perfusion solution (100  $\mu$ M). In the presence

of RP-cAMP, the current activation by 100 nM VIP was significantly reduced to  $11.9 \pm 6.9\%$  ( $P < 0.01$ ,  $n=10$  in comparison to control) (Fig. 7-5A, E).

As the adenylyl cyclase is activated by  $G_s$ , we studied the Kir6.1/SUR2B currents with forskolin, a potent adenylyl cyclase activator. Exposure of the cell to 10  $\mu\text{M}$  forskolin strongly activated the Kir6.1/SUR2B channel. Such an effect was not significantly different from the channel activation by 100 nM VIP ( $P > 0.05$ , Fig. 7-5B, E). The forskolin-activated currents also showed identical characteristics to the current activated by VIP in their I-V relationship (Fig. 7-5D in comparison with Fig. 7-3A). After the currents were activated by VIP, forskolin had no additional effect (Fig. 7-5C, E), further suggesting that the VIP and forskolin share the same intracellular signaling pathway in regulating the channel activity.

#### **7.3.4. Effects of VIP on $K_{ATP}$ channels in vascular smooth myocytes**

The effect of VIP on cell-endogenous  $K_{ATP}$  channels of the vascular smooth myocytes (VSM) dissociated acutely from mesenteric arterials. Under the same recording condition as for the HEK293 cells, the inward  $K^+$  currents were recorded from the VSMs. The VSM-endogenous current had single-channel conductance  $34.8 \pm 1.1$  ( $n=4$ ) similar to the Kir6.1/SUR2B current expressed in HEK cells ( $34.9 \pm 0.9$ ,  $n=11$ ). The currents were activated with the treatment of 100 nM VIP, further augmented by Pin (10  $\mu\text{M}$ ), and subsequently inhibited by Glib (10  $\mu\text{M}$ ) (Fig. 7-6), consistent with the Kir6.1/SUR2B channel activation observed in HEK293 cells.

To further understand whether the regulation of  $K_{ATP}$  channel by VIP affects vascular tone, we studied isometric contractions of endothelium-free mesenteric artery rings. Administration of phenylephrine (PE) led to a rapid contraction of the arterial rings which lasted over 30 min without clear desensitization. The failure of 1  $\mu\text{M}$  carbamylcholine to dilate the ring



after contraction with PE was the evidence of functional endothelial ablation (Fig. 7-7A). When VIP was applied, the rings relaxed in a concentration-dependent manner (Fig. 7-7B, D). Such a VIP-induced vasorelaxation was markedly attenuated by a pretreatment with 1  $\mu$ M Glib (Fig. 7-7C, D), indicating that the  $K_{ATP}$  channels activation play an important role in the VIP-induced relaxation.

## 7.4 Discussion

Our studies have shown that the Kir6.1/SUR2B channel is a downstream target of VIP. The channel is strongly activated by VIP with the midpoint concentration for the channel activation ~10 nM. The channel activation is likely mediated through the VIP receptor, adenylyl cyclase and PKA signaling system. Such  $K_{ATP}$  channel activation tends to hyperpolarize the cell and relaxes the vascular smooth muscle, which is consistent with our data showing that VIP produces  $K_{ATP}$  channel-dependent relaxation of the mesenteric arteries.

Several potential downstream effector molecules have been suggested for the vasodilation effect of VIP. In the isolated perfused rat heart, selective activation of VIP receptors produces vasodilation of the coronary circulation. Such a vasodilation effect can be blocked by glibenclamide but not 4-aminopyridine, suggesting that  $K_{ATP}$  channels are involved (Sawmiller et al., 2006; Tanaka et al., 1997). Another study suggests that the vasodilation effect of VIP is mediated through glibenclamide-insensitive channels (Hattori et al., 1992; Kawasaki et al., 1997). Clearly, VIP may affect vascular tones via multiple mechanisms that remain to be demonstrated, as these previous studies relied on sulfonylureas that are known to affect other ion channels as well (Ishida-Takahashi et al., 1998; Konstas et al., 2002). Using the cloned channel in the HEK expression system, we have revealed that Kir6.1/SUR2B channel is one of the downstream molecules. In the mesenteric VSMs, activation of Maxi-K channels also contributes to the vasorelaxation response to VIP, in which the cAMP-dependent signaling pathways seem to be involved (Liu et al., 1999). In the gastrointestinal system, VIP can produce relaxation of the sphincter of Oddi which can be attenuated by glibenclamide (Sari et al., 2004). Consistent with these previous studies, our results show that VIP relaxes isolated mesenteric rings, an effect that can be blocked by glibenclamide. Our studies in a heterologous expression system have indicated

that the cloned Kir6.1/SUR2B channel indeed is activated by VIP. As the Kir6.1/SUR2B channel is expressed in vascular smooth muscles (Li et al., 2003; Morrissey et al., 2005), and as the channel protein may be phosphorylated by PKA (Quinn et al., 2004), it is likely that the activation of the Kir6.1/SUR2B channel contributes to the vasodilation effect of VIP.

The  $K_{ATP}$  channels play an important role in membrane potentials and cell activity (Quayle et al., 1997; Seino, 1999). These  $K^+$  channels are inhibited by physiological concentrations of ATP, and are open when ATP level drops during metabolic stress. Such a property allows them to regulate several cellular functions during metabolic stress, including vascular tone regulation, myocardium excitability control, neuronal responses to hypoxic ischemia, insulin-secretion in pancreatic  $\beta$ -cells, and glucose uptake in striated muscles (Quayle et al., 1997; Seino, 1999; Yokoshiki et al., 1998). The Kir6.1/SUR2B channel is likely to be the major isoform of  $K_{ATP}$  channels in vascular smooth muscles, whose pharmacological properties resemble those of the native  $K_{NDP}$  channels in vascular smooth myocytes (Teramoto, 2006; Yamada et al., 1997; Yamada and Kurachi, 2004). The involvement of Kir6.1/SUR2B in vascular tone regulation has been demonstrated in both physiological and pathological conditions. Activation of these  $K_{ATP}$  channels in response to local regulators hyperpolarizes vascular smooth muscles and dilates resistance arteries leading to redistribution of the blood flow (Quayle et al., 1997), while the disruption of the *Kcnj8* (Kir6.1) or *ABCC9* (SUR2) genes in mice causes abnormalities in coronary circulation, sudden cardiac death and fatal susceptibility to endotoxemia (Chutkow et al., 2002; Kane et al., 2006; Miki et al., 2002). The vascular  $K_{ATP}$  channel is regulated by a variety of vasoactive substances especially circulating hormones and neurotransmitters. Several studies have suggested that the regulation of the vascular  $K_{ATP}$  channel is achieved via phosphorylation of the channel proteins by protein kinases that are

activated through specific intracellular singling pathways downstream to the VIP receptors (Quayle et al., 1994; Wellman et al., 1998). Therefore, the understanding of the  $K_{ATP}$  channel function in vascular tone control depends on the demonstration of the precise signal pathway underlying the channel modulation.

Several other intracellular signaling pathways may be involved in the vasodilation effect of VIP. The VIP effect has been shown to rely on the VPAC2 receptor in the coronary circulation (Sawmiller et al., 2006). Because of the lack of specific receptor blockers, we could not differentiate VPAC1 from VPAC2 in our studies. According to previous studies, the VPAC1 is expressed in the HEK293 cells (Reubi, 2000; Simmons, 1990), suggesting that the Kir6.1/SUR2B channel activation may be produced by VPAC1. However, we cannot rule out the involvement of VPAC2 as both VPAC1 and VPAC2 receptors are coupled to  $G_s$  leading to activation of adenylyl cyclase and PKA.

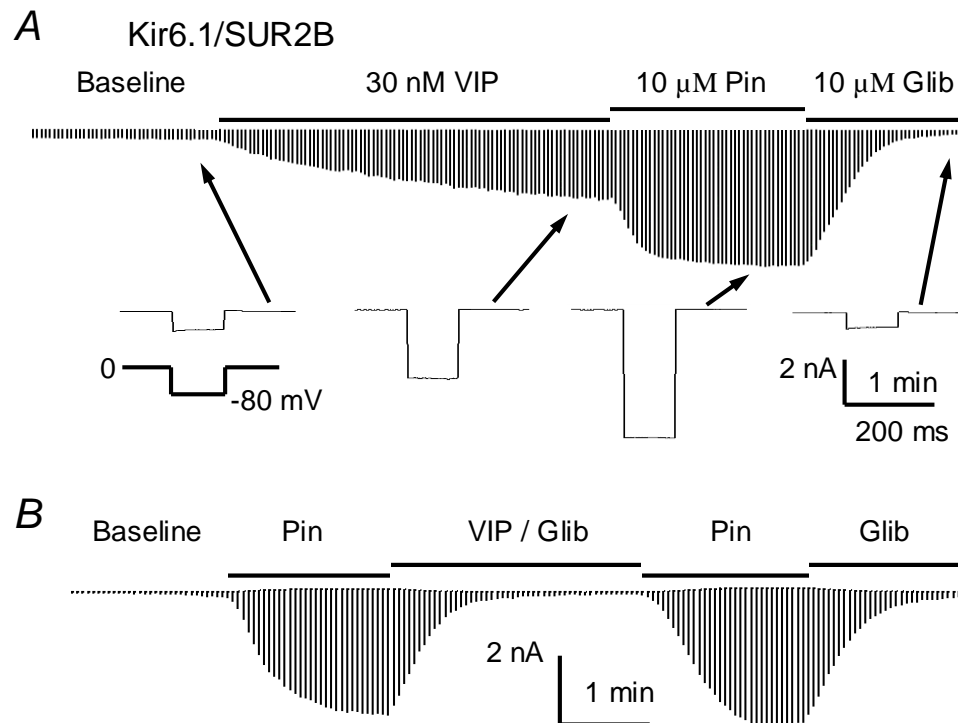
Although the VIP receptor - adenylyl cyclase - PKA system seems critical, VIP also can activate PKG pathway (Kim et al., 2006; Kurjak et al., 2001). In addition, the VIP-induced relaxation of aorta and uterine arteries has been shown to be produced by nitric oxide (NO) released from the endothelial cells (Pesic et al., 2004; Pomerleau et al., 1997). Another study however suggests that NO synthesis is not affected by VIP, while stimulation of endogenous NO production provokes VIP release from nerve terminals (Wellman et al., 1998). Since endothelium-free arterial rings were used, our results from the present study suggest that the effect of VIP on vasorelaxation is NO - independent. Because of the presence of these diverse signaling molecules, distinct intracellular signaling pathways appear to play a role in the VIP signaling targeting at perhaps different effector molecules. Thus, the demonstration of the

adenylyl cyclase - PKA - Kir6.1/SUR2B pathway in the present study is of significance in the understanding the vascular effect of VIP.

In conclusion, our results indicate that the Kir6.1/SUR2B channel is a downstream target of VIP signaling. The activation of the major vascular isoform of  $K_{ATP}$  channels can produce hyperpolarization and relaxation of vascular smooth muscles, which is consistent with the effect of VIP on mesenteric arteries. The information that we have found for the VIP signaling pathway may be useful for the manipulation of specific membrane and intracellular signal molecules in the control of vascular tones.

**Figure 7-1. Activation of the Kir6.1/SUR2B channel by VIP in HEK293 cells.** *A*, whole-cell currents were recorded from a cell transfected with Kir6.1/SUR2B. The bath solutions contained 145 mM K<sup>+</sup> so that the reversal potential of K<sup>+</sup> currents is close to 0 mV. The recording pipette was filled with the same solution with the addition of 1 mM ATP, 0.5 mM ADP, and 1 mM free Mg<sup>++</sup>. Application of VIP (30 nM) increased the whole-cell currents rapidly and reached a plateau in about 3 min. K<sub>ATP</sub> channel opener pinacidil (Pin, 10 μM) strongly augmented the currents that was potently inhibited by glibenclamide subsequently (Glib, 10 μM). Note that arrows point to where each bottom trace was taken from. *B*, the effect of VIP was totally blocked in the presence of 10 μM Glib.

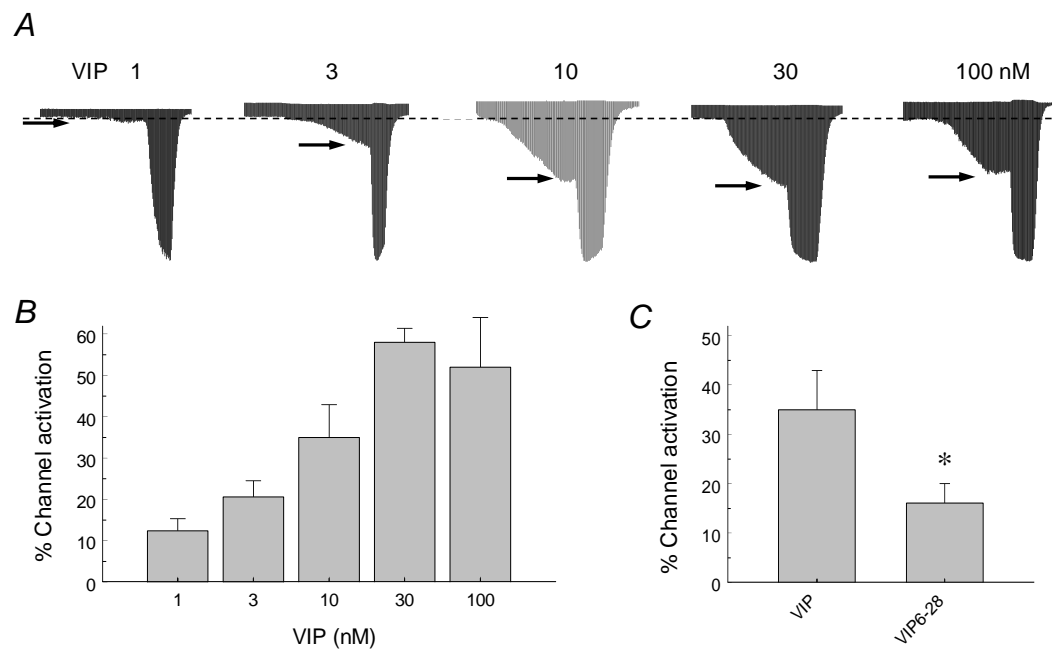
**Figure 7-1**



**Figure 7-2. Concentration dependent channel activation by VIP.** *A-B*, the activation of the Kir6.1/SUR2B channel by VIP was concentration-dependent with VIP concentration for a half current activation ~10nM. *C*, the effect of VIP (10 nM) was significantly diminished in the presence of VIP receptor blocker VIP6-28 (100 nM).

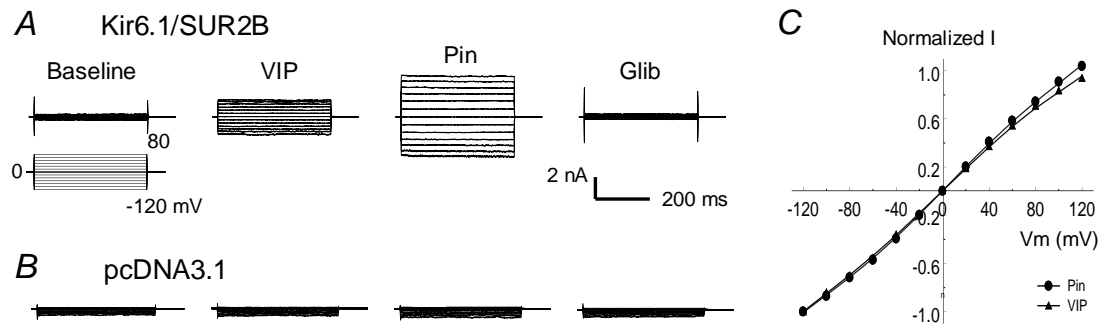


**Figure 7-2**



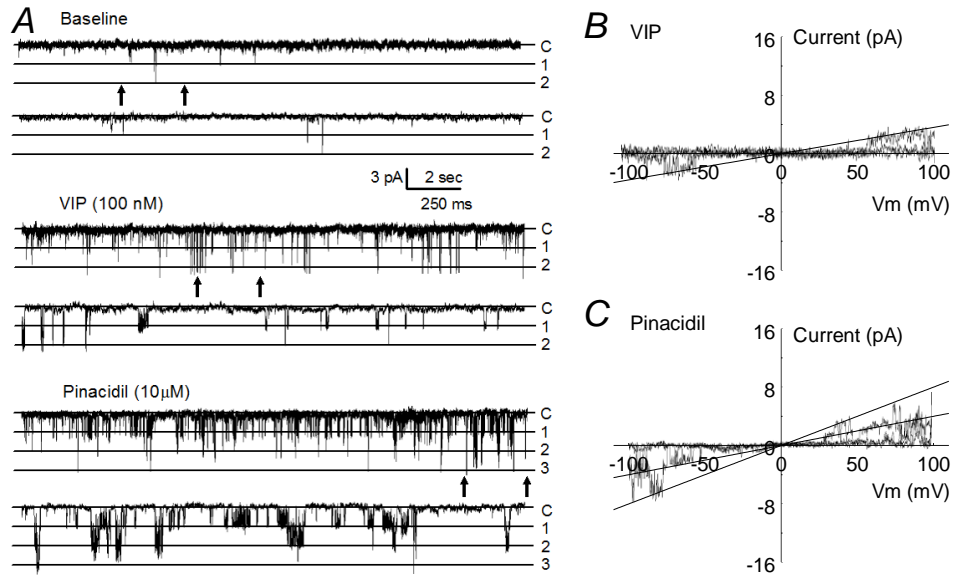
**Figure 7-3. Voltage independence.** *A*, voltage dependence was studied with step command pulses from  $-120$  mV to  $120$  mV from a holding potential of  $0$  mV. The current activation by VIP was observed across the whole voltage range in the HEK cell. *B*, similar current activation was not seen in another cell transfected with the vector alone. *C*, the I-V relationship of VIP and Pin was plotted by normalized to the current at  $-120$  mV. The I-V plots superimposed with each other almost completely indicating the effect of VIP is not voltage-dependent.

**Figure 7-3**



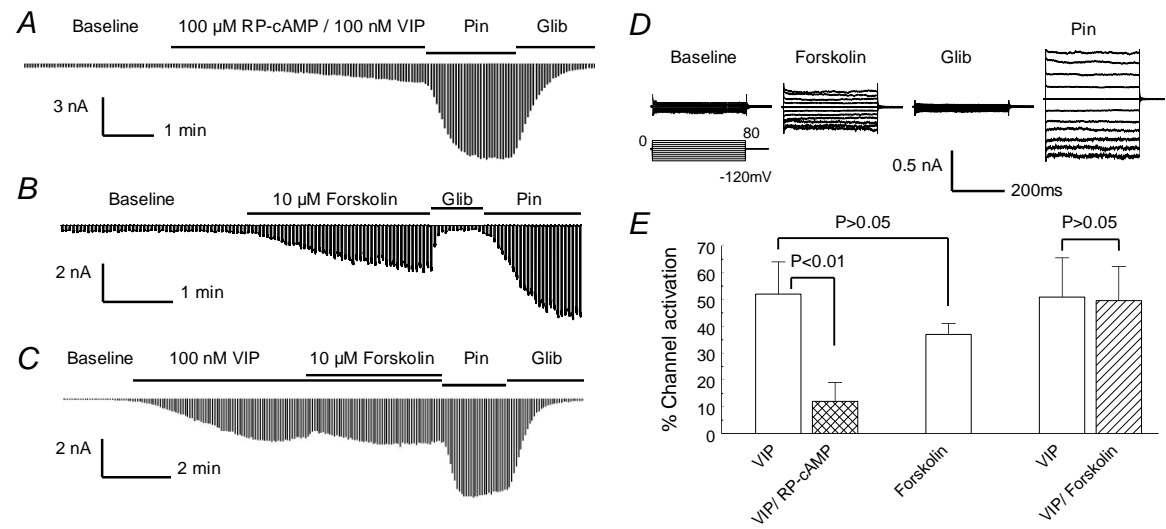
**Figure 7-4. Effect of VIP on single-channel properties.** *A*, single-channel currents were recorded in a cell-attached patch. The lower trace in each panel is an expansion from the record of upper trace between arrows. Two active channels were seen at baseline with rather low channel activity ( $NP_o$  0.001). When the cell was exposed to 100 nM VIP, the single-channel activity was augmented ( $NP_o$  0.028). The channel activity was further stimulated with 10  $\mu$ M (NP<sub>o</sub> 0.152). *B*, single channel conductance was measured in a cell-attached patch from another cell with a ramp voltage from – 100 to 100 mV. An active channel was observed with 100 nM VIP. The straight line represents a slope conductance of 36 pS. *C*, the slope conductance increased modestly, and an additional active channel was recruited in the presence of pinacidil. The slope conductance is 40 pS for both.

**Figure 7-4**



**Figure 7-5. PKA dependence of the Kir6.1/SUR2B channel activation by VIP.** *A*, RP-cAMP, a potent PKA inhibitor, was applied in both the pipette solution (200  $\mu$ M) and the perfusion solution (100  $\mu$ M). The VIP-activated Kir6.1/SUR2B currents were greatly attenuated in the presence of RP-cAMP. *B*, the Kir6.1/SUR2B channel was activated by 10  $\mu$ M forskolin, an adenylyl cyclase activator. *C*, the forskolin-activated current had similar characteristics as the VIP-activated current showing no evident voltage-dependence. *D*, after the currents were activated by VIP, forskolin had no additional effect. *E*, summary of VIP effects in the presence or absence of RP-cAMP and forskolin. The VIP-induced channel activation was significantly reduced in the presence of RP-cAMP (\*\*,  $P < 0.01$ ,  $n = 10$ ). Forskolin and VIP did not produce any significant additional activation in comparison to the effect of VIP alone.

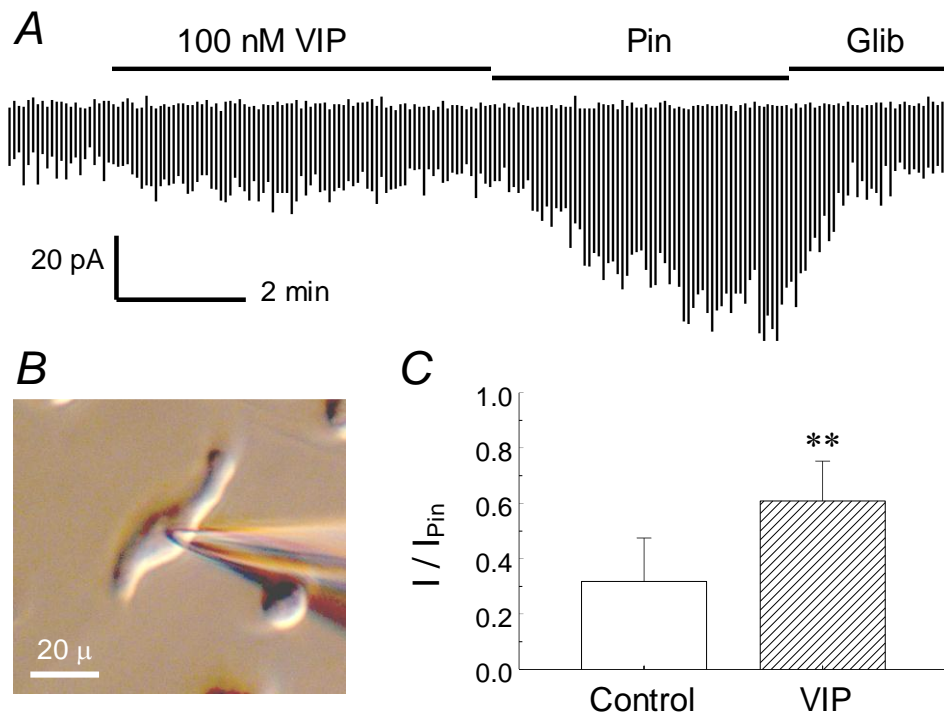
Figure 7-5



**Figure 7-6. Activation of endogenous  $K_{ATP}$  channels in vascular smooth myocytes.** *A*,  $K^+$  currents were recorded in the same condition as in Fig. 1. The currents were augmented with an exposure to 100 nM VIP. The currents were further activated by Pin and inhibited by Glib. *B*, an acutely dissociated vascular smooth myocyte with the recording pipette on the right side. *C*, the current amplitude increased significantly with VIP exposure. \*\*,  $P=0.01$ .

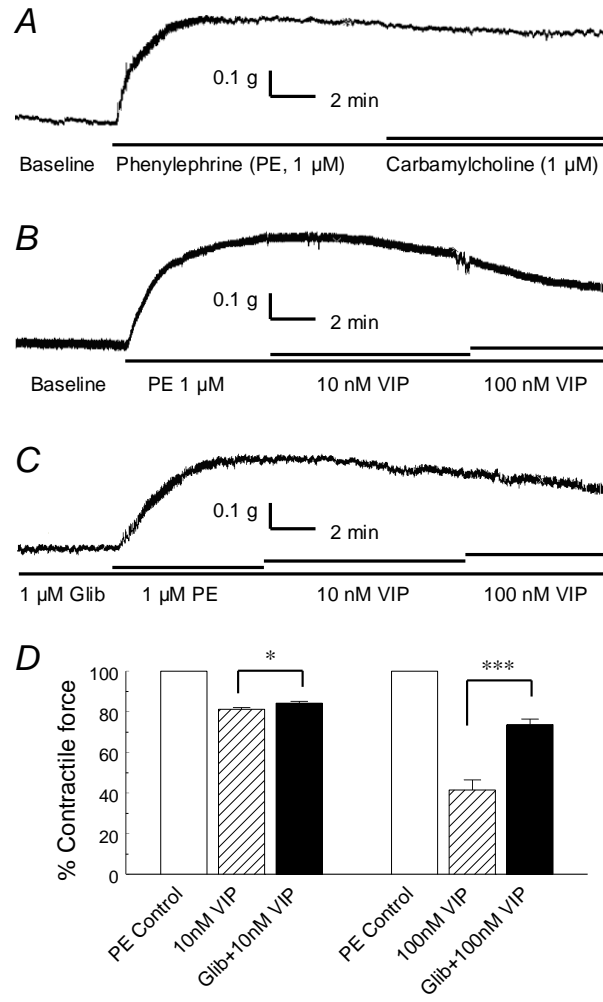


**Figure 7-6**



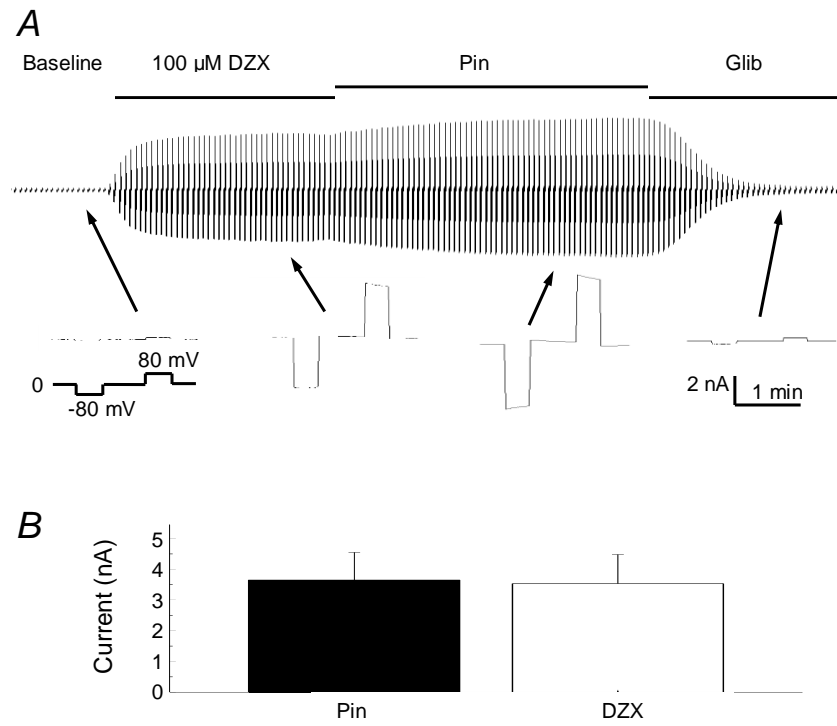
**Figure 7-7. Involvement of  $K_{ATP}$  channel in the VIP-induced vasorelaxation.** *A*, the effect of VIP was studied in an endothelium-free mesenteric arterial ring *in-vitro*. Administration of 1  $\mu$ M phenylephrine (PE) led to a strong and persistent contraction of the arterial ring. A subsequent exposure to 1  $\mu$ M carbamylcholine relaxed the ring only slightly. *B*, the application of VIP in the presence of PE dilated the artery rings in a concentration-dependent manner. *C*, such vasorelaxation effect was markedly attenuated by a 30min pretreatment with 1  $\mu$ M glibenclamide (Glib). *D*, summary of the vasodilation effect of VIP on PE-induced vasoconstriction in the presence or the absence of Glib. \*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$ .

**Figure 7-7**



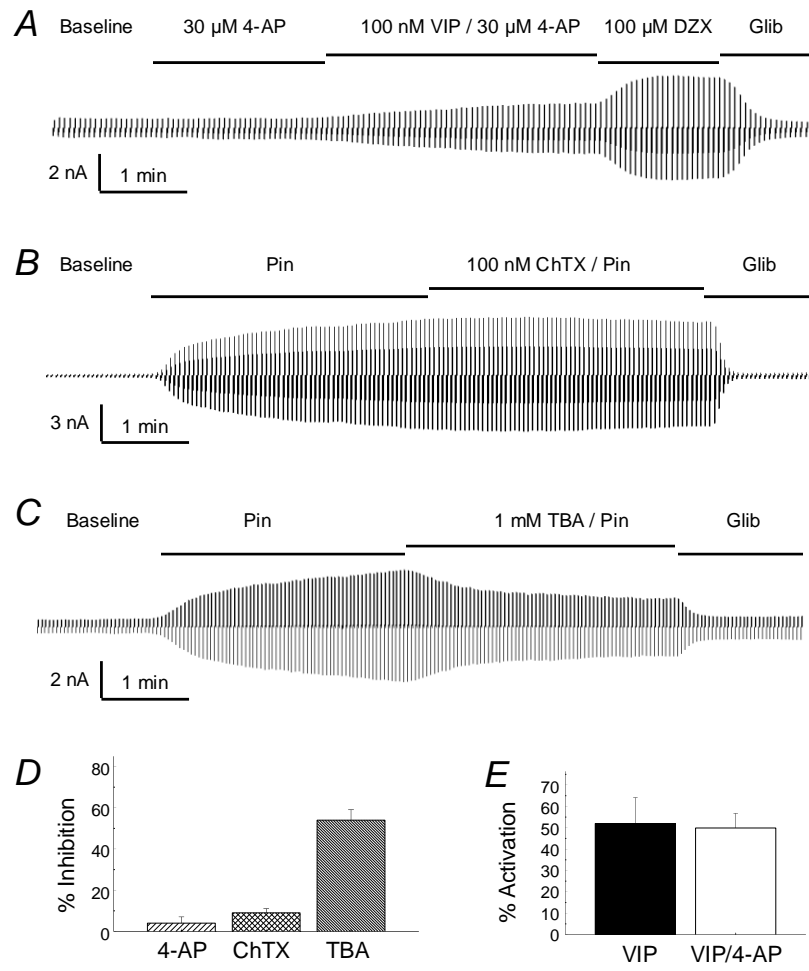
**Figure 7-8. Activation of the Kir6.1/SUR2B channel by diazoxide (DZX) and pinacidil.** *A*, DZX (100  $\mu$ M) activated the Kir6.1/SUR2B channel similarly as 10  $\mu$ M Pin, which was also inhibited by 10  $\mu$ M Glib. *B*, summary of the currents activated by DZX and Pin (n=3).

**Figure 7-8**



**Figure 7-9. The effect of different K<sup>+</sup> channel blockers on K<sup>+</sup> currents.** *A*, the pretreatment of 4-aminopyridine did not block the effect of VIP- and diazoxide (DZX)-induced currents. *B*, pin-induced K<sup>+</sup> currents were insensitive to charybdotoxin. *C*, TBA (non-selective K<sup>+</sup> channel blocker) partially blocked the Pin-induced K<sup>+</sup> currents. *D*, summary of effects of the K<sup>+</sup> channel blockers. *E*, the VIP-induced channel activation was not blocked by 4-AP treatment (n=3~5).

**Figure 7-9**



## 8 GENERAL DISCUSSIONS

### 8.1 K<sub>ATP</sub> channel modulation by PTMs

#### 8.1.1 K<sub>ATP</sub> channel modulation by PKA and PKC

Based on the distinct pore-forming subunits, K<sub>ATP</sub> channel can be separated into Kir6.1-containing channels and Kir6.2-containing channels. Both groups have been shown to be modulated by PKA and PKC signaling pathways. In general, PKA activation augments both Kir6.1 and Kir6.2-containing channels (Shi et al., 2007b; Yang et al., 2008) but the phosphorylation sites were debatable (Beguín et al., 1999; Lin et al., 2000). For example, it was reported that PKA activated the Kir6.2-containing channel (Kir6.2/SUR1) by phosphorylation of Ser372 of the Kir6.2 subunit in one report (Beguín et al., 1999). Another report supports the notion that PKA activated a variety of Kir6.2-containing channel (Kir6.2/SUR1, Kir6.2/SUR2B and Kir6.2ΔC36) but argues that Thr224, rather than the Ser372, is the phosphorylation site (Lin et al., 2000). A third report suggests that ADP plays an important role in PKA mediated channel modulations and identified Ser1448 as the phosphorylation site (Light et al., 2002). Although reports are consistent regarding the activation effect of PKA on the Kir6.1-containing channel, the site identified in our lab (Ser-1387) (Shi et al., 2007b) is different from the sites reported previously (Ser385 of Kir6.1, Thr633 and Ser1465 of SUR2B) (Quinn et al., 2004). We suggest that different NDPs used in the experimental conditions, different basal activation level and different experimental approaches, may be responsible for this discrepancy.

The effect of PKC on Kir6.1-containing channels is distinct from the Kir6.2-containing channels (Quinn et al., 2003; Thorneloe et al., 2002). PKC inhibits the activity of Kir6.1-containing channel (Kir6.1/SUR2B) (Shi et al., 2007a; Shi et al., 2008b) while it activates the Kir6.2-containing channels (Kir6.2/SUR1, Kir6.2/SUR2A, Kir6.2/SUR2B and Kir6.2ΔC26).



The phosphorylation sites of Kir6.1 have been found to consist of at least 4 serine residues located at the distal C-terminus of the Kir6.1 subunit (Shi et al., 2008b), while the phosphorylation site of Kir6.2 has been found to be at the Thr180 of Kir6.2 protein (Light et al., 2000).

Kir6.1/SUR2B channel are important vascular tone regulators. The opening of the Kir6.1/SUR2B channel contributes to vasodilation while the closure of the channel facilitates the vasoconstriction. Because many vasodilators are coupled to the PKA pathway (Yang et al., 2008) and vasoconstrictors act mostly frequently on the PKC pathway (Shi et al., 2007a), the activation of Kir6.1/SUR2B channel by PKA and the closure of Kir6.1/SUR2B channel by PKC fit very well with the actions of vasodilators and vasoconstrictors.

### **8.1.2 $K_{ATP}$ channel modulation by oxidants and thiol modulating reagents**

It is known that  $K_{ATP}$  channels are modulated by thiol-modifying reagents. The  $K_{ATP}$  channels expressed in ventricular myocytes (supposedly Kir6.2/SUR2A isoform) are inhibited by thiol modifying agents like p-chloromercuri-phenylsulfonic acid (pCMPS), 5-5'-dithio-bis(2-nitrobenzoic acid) [DTNB], and thimerosal (Coetzee et al., 1995). Interestingly, GSSG (3 mM) is unable to block the  $K_{ATP}$  channel activity in ventricular myocytes (mainly contain Kir6.2-containing channels) (Coetzee et al., 1995), which is consistent with our studies that S-glutathionylation does not have functional modification effect on Kir6.2-containing channels. Moreover, a study of cloned  $K_{ATP}$  channel (Kir6.2/SUR2B) shows that pCMPS inhibits the channel activity by targeting the Cys42 located in the N terminus of the Kir6.2 subunit (Trapp et al., 1998b).

Other studies using different thiol-modifying reagents like N-ethylmaleimide (NEM, 1 mM), DNTB (1 mM) have shown that these agents can also inhibit the  $K_{ATP}$  channels in insulin-

secreting cells (Lee et al., 1994; Matsuo et al., 1999) and muscle fibers (Weik and Neumcke, 1989). Interestingly, these inhibitory effects of thiol-modifying reagents also rely on the concentration of ATP (Lee et al., 1994; Weik and Neumcke, 1989), indicating that these cysteine modifications may have a cross-talk with the ATP/ADP sensing domains of the  $K_{ATP}$  channels.

In contrast to the thiol-modifying agents, the effect of ROS on the  $K_{ATP}$  channel activity is not consistent among different isoforms of  $K_{ATP}$  channels. ROS lower the  $K_{ATP}$  channel activity in cerebral arterioles (Erdos et al., 2004) and coronary arteries (Miura et al., 2003), but facilitates its opening in cardiac myocytes (Tokube et al., 1998) and pancreatic  $\beta$ -cells (Krippeit-Drews et al., 1999). The differential responses of  $K_{ATP}$  channels in these tissues are likely to be a result of different isoforms of  $K_{ATP}$  channels expressed in these tissues.

Although ROS mainly modify the cysteine residues in a similar way like thiol-modifying agent, studies on Kir6.2-containing channels suggest that the opening of these channels in native tissues by the application of  $H_2O_2$  is preferentially through changing the ATP/ADP sensitivity of these channels (Ichinari et al., 1996; Krippeit-Drews et al., 1999). Depending on the local environment of the target cysteine,  $H_2O_2$  may mediate different redox reactions causing the formation of either disulfide bond, S-nitrosylation, S-glutathionylation, or other intermediates. These different moieties added through these modifications may be responsible for the different effects of  $H_2O_2$  on distinct channels.

### **8.1.3 $K_{ATP}$ channel modulation by NO**

Nitric Oxide (NO) is the first identified as gaseous signaling molecule that plays a critical role in the vasodilation. It has been found that NO activates the Kir6.2/SUR2B isoform of  $K_{ATP}$  channel indirectly via the Ras/MAPK (mitogen-activated protein kinase) signaling pathway (Lin et al., 2004). Later studies show that NO activates Kir6.2/SUR1 channel via the cGMP/PKG

pathway (Chai and Lin, 2008). This regulation relies on the ROS/calmodulin signaling cascade and activates the  $K_{ATP}$  channel in a SUR1 subunit dependent manner (Chai and Lin, 2010). For  $K_{ATP}$  channel of  $\beta$ -cells, studies show that NO has a dual effect on the channel activity: at low levels NO inhibits the Kir6.2/SUR1 channel via a cGMP dependent pathway, while higher levels of NO activate the channel in a cGMP-independent manner (Sunouchi et al., 2008). NO activates  $K_{ATP}$  channels of DRG neurons in the absence of cGMP/PKG signaling compounds in excised patch condition (Kawano et al., 2009). This modulation can be reversed by DTT and blocked by thiol-alkylating agent NEM, suggesting direct S-nitrosylation (Kawano et al., 2009). Further mutagenesis studies of Kir6.2/SUR1 channel demonstrate that the NBD1 of the SUR1 subunit is the target of S-nitrosylation by NO (Kawano et al., 2009). Besides these plasma membrane  $K_{ATP}$  channels, mitochondrial  $K_{ATP}$  channels have also been studied with NO, which are directly activated (Ljubkovic et al., 2007). In our studies, we did not observe a clear effect of NO on the activity of Kir6.1/SUR2B channel. We believe that the inconsistent results might be due to the redox state of the micro-environment of the channels we studied. It would be interesting to test if  $ONOO^-$ , the molecule formed by ROS and NO, may have a modulatory effect on the Kir6.1/SUR2B channel.

#### **8.1.4 $K_{ATP}$ channel modulation by $H_2S$**

Hydrogen sulfide ( $H_2S$ ) is a gaseous messenger molecule that is generated *in vivo* from L-cysteine through the enzyme  $\beta$ -synthase (CBS) and cystathionine  $\gamma$ -lyase (CSE) (Wang, 2002). It causes vasodilation, inhibits insulin secretion, and regulates inflammation (Pearson et al., 2006). Endogenous  $H_2S$  could modulate proteins through S-sulfhydration, which converts the  $-SH$  group of cysteine to  $-SSH$  group (Mustafa et al., 2009). Many proteins are found to be regulated by  $H_2S$  (Mustafa et al., 2009) and  $K_{ATP}$  channel is among the first few being identified

(Tang et al., 2005). One study shows that H<sub>2</sub>S serves as a gasotransmitter, relaxing the mesenteric artery through the opening of vascular K<sub>ATP</sub> channels. The effect of H<sub>2</sub>S is independent the cGMP pathway mediated by NO (Tang et al., 2005). Further studies found that cloned Kir6.1/SUR1 channel can also be activated by H<sub>2</sub>S through the modulation of Cys6 and Cys26 located on the extracellular N terminal of SUR1 subunit (Jiang et al., 2010). The application of thiol modifying agents, oxidants or disulfide bond oxidizing enzymes e.g., NEM, chloramine T (CLT) disulfide or protein disulfide isomerase, does not block the effect of H<sub>2</sub>S on the channels (Jiang et al., 2010), indicating that the modulation sites for H<sub>2</sub>S are distinct from the sites that are modulated by thiol-modifying reagents. Moreover, the study of INS-1E insulin secreting cells found that endogenous H<sub>2</sub>S exists, which maintains the basal activity of  $\beta$ -cell isoform of K<sub>ATP</sub> channel (presumably Kir6.2/SUR1 channel). When the endogenously produced H<sub>2</sub>S is diminished, exogenously applied H<sub>2</sub>S (100  $\mu$ M) can further increase the K<sub>ATP</sub> current (Yang et al., 2005).

## **8.2 Ion channel S-glutathionylation**

Since oxidative stress may lead to cysteine oxidation of a variety of proteins through distinct modulation mechanisms, it is essential to test whether the cysteine oxidation is mediated through disulfide bond formation, S-nitrosylation, S-glutathionylation, sulphenic (PSOH), sulphinic (PSO<sub>2</sub>H) or sulphonic acid (PSO<sub>3</sub>H) formation using right tools. More importantly, since different experimental conditions may cause artificial oxidation, great precautions should be taken when studying S-glutathionylation using different oxidants. Using oxidants that are physiologically relevant is of critical importance to demonstrate S-glutathionylation. Because not all S-glutathionylation occurring on proteins have functional consequences, biochemical experiments should be combined with functional experiments to provide evidence for the

consequence of this modulation. Taken together, when testing the protein S-glutathionylation, several things should be taken into consideration: (1) Physiological or pathological relevance of the experimental conditions. This is important because experimental conditions are chosen to mimic the *in vivo* condition. Extreme conditions may lead to drastic protein modulation which may never occur *in vivo*. For example, micromolar concentrations of  $\text{H}_2\text{O}_2$  are better than millimolar concentrations of  $\text{H}_2\text{O}_2$  in relation to physiological relevance. Using enzyme systems like the XO/X system and NOX to produce endogenous ROS are also good choices. (2) Identification of specific reactive species and modification mechanism involved. For example, although  $\text{ONOO}^-$  is a strong endogenous RNS that have significant physiological relevance, the application of exogenous  $\text{ONOO}^-$  is likely to cause both S-nitrosylation and S-glutathionylation which need to be distinguished by other methods. (3) The combination of biochemical and functional assays. Although biochemical evidence is essential, it does not reveal non-functional S-glutathionylation. Thus, functional experiments should be combined with biochemical experiments. (4) Identification of specific cysteine residues responsible for S-glutathionylation. It has been shown previously that for a single protein, some of the cysteine residues tend to be S-nitrosylated and others are more likely to be S-glutathionylated. Therefore, identification of the specific cysteine residues may provide additional evidence for the understanding of how these modulations occur. (5) Reversibility issue. S-glutathionylation is considered as reversible reaction while some other modifications (e.g., the formation of  $\text{PSO}_2\text{H}$  and  $\text{PSO}_3\text{H}$ ) are considered irreversible. Using general reducing agent (e.g., DTT) or specific deglutathionylation enzyme (Trx or Grx) to demonstrate the reversibility would aid in distinguishing S-glutathionylation from other kinds of modifications.

### 8.2.1 Methods for the study of S-glutathionylation

Several methods for the study of S-glutathionylation have been developed. S-glutathionylation could occur on several proteins at multiple sites, thus, inducing S-glutathionylation and testing if this modification could affect the function of the protein of interest is the first critical step. GSSG (1~10 mM) is often used to induce S-glutathionylation (Chen et al., 2010; Wang et al., 2005; Yang et al., 2010) in a variety of conditions. The combined treatments of GSH (100  $\mu$ M~5 mM) with oxidants (e.g.,  $\text{H}_2\text{O}_2$ , 100~1 mM or diamide 100~1 mM) are also commonly used (Yang et al., 2010; Zmijewski et al., 2009). Besides, the application of S-nitrosoglutathione (GSNO) and  $\text{ONOO}^-$  to induce S-glutathionylation is also often practiced (Bibert et al., 2011; Figtree et al., 2009; Townsend et al., 2009; Wang et al., 2005). However, it is worth noting that these nitrogen species (10 ~500  $\mu$ M) may lead to other protein formations, e.g., S-nitrosylation. Thus, additional experiments should be performed to test for S-glutathionylation when these nitrogen species are used.

In functional studies, NEM is often used to block the S-glutathionylation (Wang *et al.*, 2005), and reactive disulfides e.g., 5-PDS, 2-PDS, DTNB are used to resemble the addition of glutathione moiety to the cysteine residues. These reagents, however, are also used for the study of S-nitrosylation (Yoshida *et al.*, 2006). The effects of reactive disulfides thus should not be directly interpreted as the effect of S-glutathionylation. Rather, the results of the reactive disulfide should be viewed as supportive evidence to other S-glutathionylation induction methods.

S-glutathionylation is considered as a reversible reaction. Reducing agents, e.g., DTT (1~20 mM) or 2-mercaptoethanol (2-ME, 1mM~10 mM), are often used to reverse the effect of S-glutathionylation. More importantly, to be physiological relevance, S-glutathionylation should

be reversed by specific enzymes including Grx, Trx, etc. Grx1 (1  $\mu$ M-50  $\mu$ M) has been suggested to be the specific de-glutathionylation enzyme, so the Grx1-mediated recovery is often essential to demonstrate the functional reversibility of S-glutathionylation of the protein of interest (Wang *et al.*, 2005; Yang *et al.*, 2010).

Biochemical experiments are also essential evidence for the demonstration of S-glutathionylation. The most popular methods to test S-glutathionylation biochemically is to use BioGEE (100  $\mu$ M~1 mM, a membrane permeable biotinylated glutathione) in immunoprecipitation (IP, or pull-down assay) and western blot experiments (Park *et al.*, 2009b; Yang *et al.*, 2010). In principle, the cells expressing proteins of interest are loaded with BioGEE (100  $\mu$ M~500  $\mu$ M) first. The cells are then challenged with oxidants, e.g., H<sub>2</sub>O<sub>2</sub> (10  $\mu$ M~1 mM) or diamide (10  $\mu$ M ~1 mM) for a time period (5 min~ a few hours). After that, the cells are lysed and streptavidin conjugated agarose beads (or magnet beads) are used to pull down the BioGEE conjugated protein complex. These pull-downed protein complex then will be run on non-reducing SDS gel and the protein of interest will be detected by the protein-specific antibodies with immunoblot (IB) (Zmijewski *et al.*, 2009). Alternatively, after the proteins are loaded with BioGEE and challenged with oxidants, the BioGEE-protein complex could be IP first with the specific antibodies against the protein of interest and then IB with streptavidin or anti-biotin antibody (Townsend *et al.*, 2009). A variation of this experiment is to avoid using BioGEE. Instead, the cells are directly challenged with oxidants and then IP with anti-GSH followed by IB with antibodies against the protein of interest (Anathy *et al.*, 2009) (or vice visa, IP with specific antibodies and IB with anti-GSH) (Figtree *et al.*, 2009). In a few cases, the use of Biotin-GSSG (2 mM) was reported, GSSG can directly cause S-glutathionylation so the challenge of oxidants

may not be needed (Bedhomme et al., 2009). With many variations available, researches should choose a method that would fit into their experimental conditions best.

Mass spectrometry (MS) is a powerful tool to detect and verify S-glutathionylation as well (Bedhomme et al., 2009; Park et al., 2009b). GSH has a molecular weight of 305 Da (Chen *et al.*, 2010). If the proteins of interest are attached with GSH, the molecular weight (MW) of the protein would show a clear shift in MS. If only one GSH is attached, then the protein MW would be shifted by 305 Da. If two are attached, then the protein MW would shift by 710 Da ( $2 \times 305$ ) and so on. By using MS, additional information regarding the number of GSH moieties attached to the target protein could be obtained. Moreover, if the proteins are digested with enzymes (e.g., trypsin or LysC) and followed by tandem mass spectroscopy (MS/MS) analysis, the specific cysteine sites that are S-glutathionylated could also be revealed (Bedhomme *et al.*, 2009; Chen *et al.*, 2010).

In addition, systemic site-directed mutagenesis is one of the golden standards to test any kinds of post-translational modification including S-glutathionylation (Chen et al., 2010). Single, double, triple or even cysteine free constructs could be generated with this method. If the proteins of interest have a homology that respond to S-glutathionylation differently, chimeras could also be created using these two homologies to determine which domains and which motifs are responsible for the modification. Combined with functional experiments or biochemical experiments, the mutant proteins could provide valuable information for the molecular basis of the modification.

The incorporated GSH moiety would interact with the surrounding amino acids and structurally change the protein conformation thus modifying the protein function. The GSH moiety can affect the protein structure in at least two ways: First, GSH is a tripeptide made of



cysteine, glutamate and glycine, so its relatively big size may have steric hindrance effect on the proteins that are normally tightly packed. Second, GSH has charged groups which may interact with the charged groups in the protein, either attracting or repelling certain amino acids to cause protein conformational changes. Structural modeling and molecular dynamics are useful tools to study how S-glutathionylation affects the protein structure. To perform the analysis, the availability of good crystallographic templates is essential. The target protein should have conserved homologies with the crystal structure that has been determined by X-ray. If the crystal structure is not available, the structural information provided by other methods including cryo-electron microscopy and nuclear magnetic resonance (NMR) spectroscopy may also be valuable. Although the resolution obtained through these methods may not be high enough compared to crystal structure, in many applications these structures are accurate enough for the study of the interaction of attached moiety (e.g., GSH) with the target proteins.

Ion channels are membrane bound proteins which are proved to be very hard to be crystalized. The limited availability of good templates for ion channels obscures the structural insight of how S-glutathionylation affects the channel functions. With the recent progress in crystal structure, studies attempting to tackle this interesting question begin to emerge. Taking the advantage of the recently crystalized chicken Kir2.2 and the bacterial Kir channel as templates, we model the Kir6.1 pore-forming subunit in its closed state to study how S-glutathionylation affects the  $K_{ATP}$  channel gating. With the model of Kir6.1 closed state, we found that the target cysteine site for S-glutathionylation (Cys176) is located in the critical region of inner helix, close to the blocking residue Phe178 (in Kir6.1) and the hinge residue Gly175 (in Kir6.1). Phe178 is suggested to block the ion conducting pathway when the channel is in its closed state. Gly175 is suggested to be the hinge, from where the channel bends to open. The

Cys176 located between Phe178 and Gly175 and its side chain faces a pocket formed by inner helix, outer helix and slide helix. When the GSH is modeled onto Cys176, we found that GSH can fit into this pocket nicely after energy minimization. To further study how this modification can affect the channel gating, we modeled the channel in its open state using an open state KirBac1.1 model determined by electron crystallography (Kuo et al., 2005). The Kir6.1 channel model in its open state shows that the inner helix undergoes a significant change, moving laterally towards the slide helix. This movement of the inner helix disrupts the binding pocket for GSH. Therefore, based on these models, we conclude that the incorporation of GSH moiety to Kir6.1 subunit prevents the channel from entering its open state thus retaining the channel close. S-glutathionylation has also been suggested to affect the ATP binding pocket of CFTR channel (Wang et al., 2005) although detailed structural insight has not been reported presumably due to the lack of accurate template for modeling. Our study demonstrates for the first time the effect of S-glutathionylation on the channel gating. We expect more insight to be gained in this field with the availability of additional channel structures.

### **8.2.2 S-glutathionylation of K<sub>ATP</sub> channel**

K<sub>ATP</sub> channels are regulated by neurotransmitters and changes in the metabolic state to regulate membrane excitability, which in turn controls insulin secretion, regulates vascular tone and protects neurons and cardiac myocytes against hypoxia, ischemia etc. K<sub>ATP</sub> channels are made of 4 pore-forming Kir (inward rectifier K<sup>+</sup> channel) subunits and 4 regulatory SUR (sulphonylurea receptors of the ATP-binding cassette) subunits. Different combinations of Kir and SUR have been found in distinct tissues including Kir6.2/SUR1 in  $\beta$ -cells, Kir6.2/SUR2A in cardiac muscle, Kir6.1/SUR2B in smooth muscles, etc. Previous studies have found that native K<sub>ATP</sub> channel can be modulated by H<sub>2</sub>O<sub>2</sub> as well as other oxidants. In heterologous expression

system, it is found that oxidants or thiol-modifying agents can also modulate these  $K_{ATP}$  channels. However, whether these modulations occur in a physiologically relevant context is not clear.

In this dissertation study, we found that the vascular  $K_{ATP}$  (Kir6.1/SUR2B) channel is inhibited by several oxidants via S-glutathionylation of the Kir6.1 subunit. Functional studies showed that the treatments of a variety of S-glutathionylation inducers including GSH/ $H_2O_2$ , (GSH:GSSG)/ $H_2O_2$ , GSSG, or GSH/diamide lead to functional channel inhibition. To test the involvements of regular disulfide bond, we studied the cells expressing Kir6.1/SUR2B channel with diamide alone. A marked inhibition effect was not seen in this condition, which suggests that the formation of disulfide bond between two cysteine residues may not be the reason for channel inhibition. We further carried out biochemical experiments by treating the cells with BioGEE together with  $H_2O_2$ . The proteins were lysed from the treated cells and streptavidin was used to pull down the BioGEE conjugated proteins. Using antibodies against Kir6.1 to probe the pulled-down proteins, we found that glutathione was incorporated to Kir6.1 subunit of  $K_{ATP}$  channel in oxidative stress conditions. We further performed mutagenesis analysis and identified Cys176 as the primary S-glutathionylation site and Cys43 as a contributing S-glutathionylation site. The Cys176 site was further confirmed by biochemical experiments. Our structural modeling suggested that the addition of GSH moiety to Cys176, which is located in the critical region for channel gating, creates a structural obstacle for the movement of inner transmembrane helices and thus prevents the channel from entering its open state. Furthermore, our studies provide evidence showing that the Kir6.2/SUR2B channel is not functionally glutathionylated and S-glutathionylation of  $K_{ATP}$  channel is SUR2B-independent.

Although our data suggest that cysteine-mediated posttranslational modification occur on  $K_{ATP}$  channel, these data do not rule out other types of cysteine modulations. NO has been shown to modulate the Kir6.2-containing channel but the underlying mechanism has been controversial (please see “ $K_{ATP}$  channel modulation by NO” for detailed description). Further research is needed to test if S-nitrosylation and other kinds of cysteine modulation mechanisms are also responsible for the inhibition or activation of different types of  $K_{ATP}$  channels.

### **8.3 ROS life-span and action range**

Since most of the ROS have a limited life span, it is likely that the redox-modification reactions occur on the proteins that are closely associated with the proteins or organelles (e.g., membrane bound NOX enzyme or mitochondria system) that generate ROS. Indeed, many membrane-associated proteins or mitochondrial proteins have been found to be redox-modified. Co-localization of the redox-modified protein TWIK-related acid-sensitive  $K^+$  channel (TASK1) with ROS generator (NOX4) has been observed. It is not known, however, if these kinds of co-localization also exist for other proteins and ROS generators. Furthermore, if the target proteins do have interactions with the ROS generator, then protein pull-down experiment and Fluorescence Resonance Energy Transfer (FRET) experiment may be used to test this hypothesis.

$H_2O_2$  has a relatively longer life span and it is suggested that  $H_2O_2$  can travel in the cytosol or across the membrane to execute its effect. However, it is also suggested that  $H_2O_2$  is a relatively weak oxidizing agent and its cytotoxic effect is from the generation of hydroxyl radicals through the interaction with a metal e.g., Fenton. Because of its stability and relatively long life span,  $H_2O_2$  is often chosen as the primary exogenous source of ROS to perform experiments. However, it is worthy to keep in mind that  $H_2O_2$  application in an experimental

condition may not exactly mimic the *in vivo* environment and caution should be taken to interpret the data. Therefore, using enzymatic systems or enzyme activators to manipulate the endogenous redox system may serve as an alternative. In pathological conditions,  $H_2O_2$  and related ROS may be constantly produced. The long-lasting production of ROS may cause a gradual inhibition of the vascular  $K_{ATP}$  channel. When the channel inhibition reaches a significant level, a cascade of events may be triggered, leading to extensive depolarization, excessive ROS production, structural damages to the cell, and disruption of cellular function. Therefore, early termination of the cascade of events is necessary, which may be achieved by activation of the  $K_{ATP}$  channels at the early stage of the pathological conditions using several endogenous regulators such as VIP.

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## APPENDICES: PUBLICATIONS

- 1 **Yang Y\***, Shi W\*, Chen X\*, Cui N, Konduru AS, Shi Y, Trower TC, Jiang C. Molecular basis and structural insight of vascular K<sub>ATP</sub> channel gating by S-glutathionylation. *J Biol Chem* 2011, 286: 9298-307
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- \*, equal contributions.